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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Thaddeus P. Dryja et al.
Serial No.:
Filed : Herewith
Title : DIAGNOSIS OF RETINOBLASTOMA

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Box PATENT APPLICATION

Commissioner of Patents and Trademarks
Washington, DC 20231

REQUEST FOR FILING DIVISIONAL APPLICATION

This is a request for filing a divisional application under 37 C.F.R. 1.53(b), of pending prior application Serial No. 08/255,572, filed on June 8, 1994 by Thaddeus P. Dryja, Stephen Friend, and David W. Yandell for DIAGNOSIS OF RETINOBLASTOMA.

1. Enclosed is a true copy of the above identified prior application. The true copy of the prior application is as follows: 44 pages of specification; 5 pages of claims; one page of abstract; and 18 informal sheets of drawings.

Express Mail No.: EJ695382670US
Date of Deposit: 8/31/99. I hereby certify under 37 C.F.R. § 1.10 that this correspondence is being deposited with the United States Postal Services as "Express Mail Post Office to Addressee" with sufficient postage on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231
By: *Eric J. Roberts*

09387158-023199

2. The original claims in the prior application are claims 1-21. The filing fee, based on the original claims in the prior application, is calculated below:

CLAIMS AS FILED				
	Number Filed	Number Extra	Rate	Basic Fee
				\$760
Total Claims	22 - 20 =	2 x	\$ 18 =	36
Independent Claims	13 - 3 =	10 x	\$ 78 =	780
Multiple Dependent Claims	1		\$260 =	260
Total Filing Fee			=	\$1,836

2. A copy of the "Revocation and Substitution of Power of Attorney by Assignee Under 37 CFR §§ 3.71 and 3.73" which was filed on August 6, 1999 in the prior application is enclosed.

3. Enclosed are 18 sheets of informal drawings for use in this application.

4. Please amend the specification by inserting before the first line the sentence:
--This is a divisional of copending application Serial No. 08/255,572, filed June 8, 1994, which is a continuation of Serial No. 07/951,342, filed September 25, 1992, now abandoned, which is a continuation of application Serial No. 07/728,756 filed July 8, 1991 now abandoned, which is a continuation of application Serial No. 07/300,667 filed January 23, 1989 now abandoned; which is a continuation in part of U.S. Serial No. 07/146,525 filed January 21, 1988; which is a continuation in part of U.S. Serial No. 06/895,163 filed August 11, 1986.--

5. With respect to the prior application from which this application claims benefit under 35 U.S.C. 120, the inventors named in this application are the same as those named in the prior application.

6. The prior application is assigned of record to Massachusetts Eye and Ear Infirmary, a Massachusetts corporation having a place of business at Boston, Massachusetts, by virtue of an assignment submitted to the Patent and Trademark Office for recording on April 24, 1989 at Reel 5081, Frame 0968 and 0969; and is assigned of record to Whitehead Institute, a

Delaware corporation having a place of business at Cambridge, Massachusetts, by virtue of an assignment submitted to the Patent and Trademark Office for recording on April 24, 1989 at Reel 5081, Frame 0970.

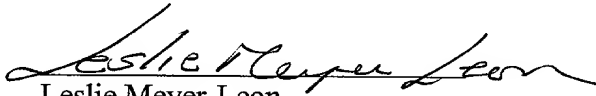
7. Enclosed is a check in the amount of the filing fee of \$1,836.00.

8. The Commissioner is hereby authorized to charge any fees which may be required by this paper, or credit any overpayment, to Deposit Account No. 50-0311, Ref. No. 19100-021. A duplicate copy of this sheet is enclosed.

9. The power of attorney in the prior application is to Leslie Meyer-Leon, Reg. No. 37,381. Address all future communications to Leslie Meyer-Leon at Mintz, Levin, Cohn, Ferris, Glovsky and Popeo, P.C., One Financial Center, Boston, Massachusetts 02111 (direct telephone number 617-348-4432).

Respectfully submitted,

Date: Aug 31, 1999


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JOINT

APPLICATION

FOR

UNITED STATES LETTER PATENT

DIAGNOSIS OF RETINOBLASTOMA

Background of the Invention

This application is a continuation-in-part of Dryja et al. U.S.S.N. 146,525, filed January 21, 1988, which is a continuation-in-part of Dryja et al. U.S.S.N. 895,163, filed August 11, 1986, both hereby incorporated by reference.

This invention concerns the retinoblastoma gene and methods for detecting and treating patients afflicted with a defective retinoblastoma gene.

Retinoblastoma is a neoplastic condition of the retinal cells, observed almost exclusively in children between the ages of 0 and 4 years. It affects between 1 in 34,000 and 1 in 15,000 live births in the United States. (L.E. Zimmerman, 1985, Retinoblastoma and retinocytoma, In W.H. Spencer (ed.), Ophthalmic Pathology: an Atlas and Textbook, Vol. II, Philadelphia: W.B. Saunders Co., pp. 1292-1351.) If untreated, the malignant neoplastic retinal cells in the intraocular tumor travel to other parts of the body, forming foci of uncontrolled growth which are always fatal. The current treatment for a retinoblastoma is enucleation of the affected eye if the intraocular tumor is large; for small intraocular tumors, radiation therapy, laser therapy, or cryotherapy is preferred. There is no known successful treatment for metastatic retinoblastoma. As with most cancers, morbidity and mortality are reduced if diagnosis can be made early in the course of the disease.

least 15 bases, encoding the Rb gene. The invention also concerns cells transformed with this nucleic acid, isolated polypeptides encoded by this nucleic acid, and antibodies to this polypeptide, or to naturally occurring retinoblastoma polypeptide. Retinoblastoma polypeptide is the polypeptide encoded by the Rb gene. Further, the invention concerns a composition, suitable for treating a human having a defective Rb gene, containing retinoblastoma polypeptide, or a fragment thereof, in a pharmacologically acceptable carrier.

The invention also features methods of screening human patients to determine those not at risk of developing retinoblastoma and thus not requiring conventional examinations to be performed. This screening involves, for example, comparing nucleic acid of a patient with purified nucleic acid encoding a human Rb gene, or fragments thereof.

Thus in various aspects, the invention features methods of analyzing the predisposition of patients to retinoblastoma which involves detecting large and small deletions or point mutations in the retinoblastoma gene, or detecting the co-inheritance of such defects with specific restriction fragment length polymorphisms (RFLPs), or detecting the presence or absence of a normal or defective retinoblastoma gene by hybridizing a nucleic acid sample from the patient with a probe specific for the retinoblastoma gene, and determining the ability of the probe to hybridize to the nucleic acid. The

lack of hybridization to the nucleic acid indicates the presence of a large deletion in the gene. A probe specific for the retinoblastoma gene may be hybridized to fragments separated by a defined physical property from a sample of a patient, the hybrids of the probe and the fragments detected, and the hybrids compared to hybrids detected from the hybridization of the probe and separated nucleic acid fragments from a normal retinoblastoma gene. The absence of hybrids or presence of hybrids of a smaller size compared to a normal patient is an indication of large deletions in the retinoblastoma gene of the patient. Preferably, the probe specific for the retinoblastoma gene is the cloned DNA in p4.7R, or a fragment thereof; and the defined physical property is molecular weight.

Small deletions or point mutations can be detected by determining the nucleotide sequence of a retinoblastoma allele from a patient, and comparing the nucleotide sequence with the nucleotide sequence of a retinoblastoma allele, or subregion thereof, from a person not afflicted with retinoblastoma; or by detecting mismatches between a nucleic acid sample from a patient and a probe specific for the retinoblastoma gene from a person not afflicted with retinoblastoma. The co-inheritance of specific genetic polymorphisms with the retinoblastoma gene may be an indication of the predisposition of a patient to retinoblastoma. According to this method, nucleic acid

fragments are generated from a sample of the patient, the fragments are separated according to a defined physical property of the fragments (e.g., molecular weight), a detectable probe specific for the retinoblastoma gene is hybridized to the fragments, hybrids of the probe and the fragments are detected, and the hybrids are compared to hybrids detected from the hybridization of the same probe and separated nucleic acid fragments from a sample of a parent of the patient.

In another aspect, the invention features the use of an isolated normal human retinoblastoma gene to synthesize Rb polypeptide for use in the treatment of individuals determined to have a defective Rb allele.

In yet another aspect, the invention features a method of detecting the presence of the retinoblastoma polypeptide in a tumor sample from a human patient, by producing an antibody to the retinoblastoma polypeptide, contacting the antibody with the tumor sample, and detecting immune complexes as an indication of the presence in the tumor sample of the retinoblastoma polypeptide. The absence of the polypeptide indicates that the tumor is caused by a defect in a retinoblastoma allele. This procedure would preferably involve contacting a tumor sample from a human patient with an antibody (e.g., monoclonal antibody) which specifically reacts with the retinoblastoma polypeptide, or a fragment thereof, and determining whether the antibody binds to cells of the tissue

specimens. The absence of immune complexes is an indication that the tumor was the result of a defective retinoblastoma allele.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The description of the preferred embodiments of the invention will follow the brief description of the drawings given below.

Drawings

Fig. 1 is a diagrammatic representation of a restriction map of the insert in the clone p4.7R.

Fig. 2 is a diagrammatic representation of a restriction map of the genomic locus of the retinoblastoma gene.

Fig. 3 is a diagrammatic representation of the vectors p2AR3.8 and p2AR0.9 of the invention.

Fig. 4 is a scale map of the normal retinoblastoma gene.

Fig. 5 (5-1 through 5-3) is a nucleic acid sequence of a cDNA of the normal retinoblastoma gene, with flanking regions.

Fig. 6 (6-1 through 6-9) is a nucleic acid sequence of exons of the normal retinoblastoma gene, with flanking regions.

Fig. 7 is a restriction map of the retinoblastoma gene, showing the locations of DSPs.

Fig. 8 is a gel and a diagram showing the inheritance of the polymorphism RB1.3 in a retinoblastoma-prone family.

Fig. 9 is a diagram showing the segregation of the DSP RB1.3 in three families with hereditary retinoblastoma.

Retinoblastoma Polypeptide

The Rb polypeptide is the specific amino acid chain encoded by the nucleic acid sequence of the normal retinoblastoma gene. The Rb polypeptide of this invention includes: (1) naturally occurring retinoblastoma protein; (2) synthetically produced retinoblastoma polypeptide; and (3) retinoblastoma polypeptide produced from purified nucleic acid (e.g., cDNA or genomic DNA) via an in vitro expression system. Also included are biologically active fragments of Rb polypeptide which either have a biological activity of naturally occurring Rb polypeptide, or include an epitope of this polypeptide and thus are suitable for production of Rb-specific antibodies.

Retinoblastoma Gene

The Rb gene is that distinct nucleic acid sequence in the human genome, the absence or mutation of which predisposes one to retinoblastoma. The purified nucleic acid sequence encoding the retinoblastoma gene can be carried on vectors which can be propagated in cells. For the purposes of this invention, purified nucleic acid encoding the Rb gene is defined as nucleic acid isolated from its natural environment

(e.g., cDNA or a fragment of genomic DNA) which hybridizes specifically to the retinoblastoma gene under hybridizing conditions. An example of purified nucleic acid which encodes the retinoblastoma gene, and is carried on a vector, is the cDNA clone p4.7R. This clone was obtained in the following manner.

cDNA

The human DNA probe pH3-8, isolated from a human chromosome 13 lambda phage library (M. Lalande et al., 1984, Cancer Genet. Cytogenet., Vol. 13, pp. 283-95), was used in a chromosome walking technique to isolate and map 30 kilobases (kb) of genomic DNA surrounding the H3-8 sequence. One fragment generated by this technique, named p7H30.7R, was found to recognize a DNA sequence in the mouse genome as well as one within human chromosome 13 (T.P. Dryja et al., 1986, Proc. Natl. Acad. Sci. USA, Vol. 83, pp. 7391-94). The homology of p7H30.7R to both human and mouse DNA suggested that p7H30.7R contains coding sequences of a structural gene.

To test this possibility, p7H30.7R was radiolabeled and used to probe a Northern blot of RNA isolated from three retinoblastoma tumors and an adenovirus 12-transformed human embryonic retinal cell line (Vaessen et al., 1986, EMBO Journal, Vol. 5, pp. 335-). The p7H30.7R probe hybridized to an RNA transcript of approximately 4.7 kb from the retinal cell line, but did not hybridize to any RNA transcripts from the

three tumor samples.

Subsequently, RNA isolated from the adenovirus-transformed retinal cell line was used to construct a cDNA library. This library was screened with the labeled p7H30.7R probe. Several cDNA clones were isolated which had similar restriction maps. The longest of these, p4.7R, contained 4.7 kb of DNA. The restriction map of the insert in the clone p4.7R is shown in Fig. 1.

The p4.7R clone was used to screen RNA transcripts isolated from four retinoblastomas, an osteosarcoma, and the adenovirus-transformed retinal cells. In a Northern blot analysis of isolated RNA's, the p4.7R probe cross-reacted with a 4.7 kb transcript in the transformed retinal cells which was not present in the four retinoblastoma and one osteosarcoma cell samples.

Genomic DNA

Clones containing genomic DNA including the retinoblastoma gene were isolated in the manner described below. Recombinant bacteriophage libraries containing human genomic DNA fragments inserted in the lambda phage cloning vector EMBL-3 were constructed according to published methods (Seed et al., 1982, Gene, Vol. 19, pp. 201-209). Those recombinant bacteriophage which contain fragments of the retinoblastoma gene were initially detected by hybridization of the bacteriophage plaques with p4.7R.

Thirty-six distinct recombinant bacteriophage that contain overlapping human genomic DNA fragments were isolated. Selected bacteriophage were plaque-purified and amplified, and the restriction map of each phage insert was determined by the method of Rackwitz et al., Gene, Vol. 30, pp. 195-200.

With these bacteriophage a restriction map of a region that spans approximately 200 kb was constructed, shown in Figs. 2 and 4. All of the known sequences present in the mRNA from the retinoblastoma gene are present in this cloned region. In aggregate, the human DNA sequences in this set of bacteriophage represent the chromosomal segment of the Rb gene. In Fig. 2, the vertical marks above the map represent the location of HindIII sites, and the vertical marks below the map represent the location of EcoRI sites. The boxed areas represent HindIII fragments which contain sequences found in the cDNA (exons). Each double-headed arrow beneath the map represents a distinct recombinant bacteriophage clone. Fig. 4 shows all the recognition sites of the six restriction enzymes Hind III, EcoR I, Xba I, Sac I, Sac II, and BamH I (New England Biolabs, Inc.). Restriction endonuclease fragments that contained exons were identified by their hybridization with cDNA clones or synthesized oligonucleotide sequences based on cDNA sequence. These restriction fragments were subcloned in the plasmid vector Bluescribe (Stratagene, San Diego, CA). A total of 24 distinct plasmids were subcloned in this manner.

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The number and size of each exon was determined by iterations of the following procedure. First, an oligonucleotide was synthesized that corresponded to the first 20 nucleotides of the cDNA sequence. Using this oligonucleotide as a primer, the plasmid with a genomic insert containing the most 5' exon was sequenced. The resultant sequence was aligned with the cDNA sequence to determine the length of the first exon; the point at which the plasmid and cDNA sequences diverged marked the beginning of the first intron. This exon and the flanking regions were further sequenced using synthetic oligonucleotide primers to generate a continuous nucleotide sequence composed of 5' promoter sequence, exon 1, and the beginning of intron 1. The second and subsequent exons were defined by synthesizing sequencing primers corresponding to the next 20 nucleotides of cDNA sequence that had not been previously assigned to an exon. All exons and the immediately adjacent flanking intron sequences were sequenced in both sense and antisense directions.

The dideoxynucleotide chain termination method of sequencing was carried out using the enzyme Sequenase (United States Biochemical Corporation, Cleveland, Ohio) according to protocols supplied by the manufacturer. The intron region downstream of exon 20 could not be sequenced by this method, due to an unusually problematic repeated sequence in this region that caused a series of 45 stops (bands appeared in all

four lanes of the sequencing gel). To resolve this region, sequencing reactions were carried out with Taq polymerase (Perkin-Elmer/Cetus). This enzyme allowed for the polymerization to be performed at 68°C and resolved the bases in this region.

All sequence data were analyzed and screened for overlapping regions using the sequence analysis program Microgenie Sequence Software (Beckman, Palo Alto, CA).

The position of each exon within the restriction map of the gene was determined by hybridization of cDNA fragments or synthetic oligomer sequences to recombinant bacteriophage DNA that had been digested with various restriction endonucleases. The precise location of most exons was subsequently deduced when recognition sequences of endonucleases were identified within the intron-exon sequence and correlated with the map. The position of each of the remaining exons was arbitrarily placed in the center of the smallest restriction fragment to which it hybridized.

The organization of the 27 exons along the genomic map of the retinoblastoma gene is illustrated in figure 4. This figure details the recognition sites for 6 restriction endonucleases and the position of the exons relative to these sites. Exons 1, 2, 3, 6, 9, 10, 13, 21, 22, 23, 24, 25, 26, and 27 have been precisely localized on this map. The other exons were mapped within small restriction fragments and are

illustrated in the middle of these fragments. Exons 11, 17, and the cluster of exons 14-16 were mapped by this technique with uncertainties of not more than 2.0 kb. The remaining exons (exons 4, 5, 7, 8, 12, 18, 19, and 20) were all mapped to within 0.8 kb of their true locations. For reference, this map also shows the position of several naturally occurring restriction fragment length polymorphisms.

Fig. 6 (6-1 through 6-9) shows the sequence flanking and including each exon. The exons range in size from 31 nucleotides (exon 24), to 1973 nucleotides (exon 27). The shortest intron sequence was found to be 80 nucleotides long and is located between exons 15 and 16, whereas the largest spans approximately 70.5 kb between exon 17 and 18. All of the intron donor and acceptor splicing sites comply with the GT-AG splice junction rule. Our methods of sequencing proved more accurate than previous reports in defining the exact number of exons comprising the retinoblastoma gene.

The first exon and the region immediately 5' to this exon are very G-C rich, which is a characteristic of promoter regions. This region contains 9 possible Hpa II restriction sites, is composed of 66% C+G nucleotides and does not exhibit CpG suppression. These criteria are indicative of a HTF island. This promoter region contained some nucleotides that could not be resolved in either the sense or antisense direction using either Sequenase or Tag polymerase.

Presumably, this was due to secondary structure that forms in this promoter region.

Analysis of the sequence approximately 30 nucleotides upstream of the transcription initiation site defined by Lee et al. (1987b) does not reveal a TATA box that is found in other promoter regions. This suggests that either the previously published initiation site is not in fact correctly defined, or that the retinoblastoma gene lacks the prototypical TATA and CAAT boxes of promoter regions. Further analysis of the sequence 5' to exon 1 reveals a possible TATA box at base pair #-274, labeled base pair #122 in figure 6-1. Homology for the seven base region is only 57%, yet the first four bases T-A-T-A, which are the bases most frequently conserved, are 100% homologous. A possible capping site, CAC, is located 14 bases away and again 49 bases away. CAAT boxes were not identified although the region is generally G-C rich.

The intron sequence that flanks the 3' side of exon 20 consisted of 21 consecutive repeats of the sequence TTT(T)C that together span 87 nucleotides. The number of repeat units can vary between different individuals and the alleles determined by the number of repeats behave like a heritable DNA polymorphism.

A computer search of the sequence data identified several intron regions homologous to Alu repetitive sequences. Alu repeats were located in the following regions: (1)

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downstream of exon 2, between bp 492 and bp 704 according to the numbering scheme in Figure 6; 2) upstream of exon 9, between bp 19 and bp 117; 3) downstream of exon 11, between bp 504 and bp 680; 4) the intron sequence flanking both sides of exon 14 between bp 132 and bp 270 and between bp 420 and bp 741; and, 5) upstream of exon 17, between bp 36 to bp 210. The Alu sequence located downstream of exon 2 contains two internal sequences that are highly conserved in Alu repetitive sequences. The first is a sequence (GAGGCNGAGC) corresponding to the T-antigen binding sequence of the SV40 replication origin. The second is a symmetrical sequence (CCAGCCTGG) of no known function. This short symmetrical sequence is also present in both of the Alu sequences on either side of exon 14. Exons 14 and 15 are separated by a short intron that is almost entirely composed of Alu sequence, suggesting that exons 14 and 15 were possibly at one time a single exon and were divided by the insertion of an Alu element during evolution. This Alu sequence may have been directed to this position by the other Alu sequence located on the 5' end of exon 14 because retroposons have a tendency to integrate adjacent to one another.

The 3' end of the retinoblastoma gene contains the usual polyadenylation signal sequence, AATAAA. One sequence (TGTGTTCT) located 32 bases downstream of this hexamer is equivalent to the conserved downstream consensus sequence.

(YGTGTTY) described by McLauchlan et al. (1985). This sequence and surrounding bases compose the "G/T cluster" generally found in a region 30 nucleotides downstream of the polyadenylation signal sequence (Birnstiel et al., 1985).

The p4.7R probe also was used to screen genomic DNA isolated from the tumors of 50 unrelated individuals (40 retinoblastomas, 8 osteosarcomas, and 2 undifferentiated tumors of unknown cellular origin arising in patients with hereditary retinoblastoma), as described in more detail below. These DNA samples were digested with HindIII and analyzed by Southern blot hybridization using radiolabeled p4.7R as the probe. This analysis revealed three types of deviant patterns of the genomic DNA restriction fragments: totally absent fragments, representing apparent homozygous deletions; under-represented fragments, representing apparent heterozygous deletions; and fragments of altered size, reflecting either partial deletion or an alteration of a restriction site. . At least 30% of the tumor DNA's exhibited one of these abnormalities. In comparison, Southern blot analysis of leucocyte DNA from 18 normal individuals showed a uniform pattern of restriction fragments.

Use

The cDNA and genomic sequences, e.g., those in p4.7R, can be used, according to the invention, to screen individuals for the presence of a mutated allele of the Rb gene. This

screening procedure will allow individuals having a risk of developing retinoblastoma--because of family history or a previous incidence of retinoblastoma in one eye--to determine the need for routine testing by the current ocular examination procedure. Only if the screening procedure determines that the individual possesses a mutant Rb allele will the examination procedure need to be conducted on a regular basis. Those with two normal Rb alleles can discontinue examination, as the risk of developing retinoblastoma in an individual with two normal copies of the Rb gene is approximately 1 in 20,000, or 0.005%, compared to a risk of 80%-90% if an individual has an Rb allele containing a mutation sufficient to inactivate the allele. Thus, a substantial percentage of individuals who are currently examined regularly are not actually at a greater risk than the general population: neither a family history of nor a previous incidence of retinoblastoma is conclusive evidence that an individual has the genetic predisposition to the disease. Therefore, such individuals, actually carrying two normal copies of the Rb gene, have been repeatedly undergoing the expensive and traumatic ocular examination procedure needlessly.

The screening procedure according to the invention includes: (1) testing a nucleic acid sample of a patient for large deletions in the Rb gene locus; (2) testing a nucleic acid sample of a patient for small deletions or point mutations in the Rb gene locus; and (3) testing a nucleic acid sample of

a patient for RFLPs linked to the Rb gene locus.

Detection of Large Deletions in the Rb Gene

The availability of DNA probes from the Rb gene provides a means of directly detecting genetic lesions that create retinoblastoma-predisposing alleles. Suitable probes include the entire normal retinoblastoma gene sequence, or fragments thereof consisting of 15 or more bases encoding a specific portion of the retinoblastoma gene. When performed by Southern blot and dot blot procedures, this analysis is generally limited to the study of those lesions that create gross structural changes in the Rb gene, such as deletion of many hundreds of base pairs.

The DNA for a Southern Blot or dot blot analysis is isolated from peripheral leucocytes or, if the patient has had a tumor in one eye, from the tumor. To examine leucocyte DNA, a 10 ml blood sample is obtained from the individual, and the genomic DNA is isolated from the leucocytes in the sample, according to standard techniques. This DNA is digested with a restriction endonuclease (e.g., HindIII), and the resulting fragments are separated on an agarose electrophoresis gel according to a physical property such as molecular shape or molecular weight. For the purposes of this invention, molecular shape is defined as the structural configuration of the molecule (e.g., linear, circular, double-stranded or single-stranded). The DNA in the gel is transferred to a

nitrocellulose filter by blotting. The filter is then probed with, e.g., radiolabeled p2AR3.8 and, separately, p2AR0.9, containing subfragments from p4.7R obtained by EcoRI digestion. (The diagrams of the vectors p2AR3.8 and p2AR0.9 are shown in Figure 3.) In order to more precisely define the location of any abnormalities detected, two or more subfragment probes are used separately rather than the entire p4.7R insert probe. The autoradiograms of the probed filter generate the data necessary to construct a restriction map of the Rb locus in the somatic or tumor DNA of the tested individual.

This restriction map is compared with a control restriction map, determined by using the same restriction enzymes for digestion and the same probe. A suitable control is DNA obtained from an adenovirus-transformed retinal cell line or leucocyte DNA from a set of normal individuals. If the tested individual has an Rb allele containing a significantly large deletion, a restriction map of his DNA, compared with the control, will contain an additional band or bands, and/or a band or bands that have lost 50% of their intensity, caused by a change in the size, or total elimination, of one or more restriction fragments by the deletion in one allele at the Rb locus.

This screening procedure by Southern analysis will detect the existence of Rb alleles which have large deletions and are thereby non-functional. If this analysis indicates

that the tested DNA from an individual has a restriction map which is different from the control map, there is a high probability that the individual contains a non-functional, mutant Rb allele. The individual must be monitored closely thereafter for the development of retinoblastoma.

If the test restriction map appears identical to the control, a different screening procedure can be performed to determine if the individual possesses an Rb allele having a small deletion or point mutation. Small deletions and point mutations may be sufficient to inactivate the allele, but not prevent hybridization with a probe. An example of this screening procedure is outlined below.

Detection of Other Mutations in the Rb Gene

To examine a DNA sample of an individual for small deletions or point mutations in the Rb locus, both homologs of the Rb gene from said individual are cloned. The cloned alleles then can be tested for the presence of nucleic acid sequence differences from the normal allele, e.g., as represented by p4.7R, by one of the following two methods: (1) the nucleotide sequence of both the cloned alleles and p4.7R are determined and then compared, or (2) the RNA transcripts from p4.7R are hybridized to single stranded whole genomic DNA from an individual to be tested, and the resulting heteroduplex is treated with Ribonuclease A (RNase A) and run on a denaturing gel to detect the location of any mismatches. In

The alleles of the Rb gene in an individual to be tested are cloned using conventional techniques. A common method, for example, employs the bacteriophage vector EMBL3 (Frischauf et al., 1983, J. Mol. Biol., Vol. 170, pp. 827-). A 10 ml blood sample is obtained from the individual. The genomic DNA isolated from the cells in this sample is partially digested with MboI to an average fragment size of approximately 20 kb. Fragments in the range from 18-21 kb are isolated. The resulting MboI-ended fragments are ligated into the EMBL3 vector DNA which has been completely digested with BamHI, treated with alkaline phosphatase, and heated to 68°C for 10 minutes to disrupt the cohesive ends. This ligation mix is used in an in vitro lambda packaging reaction, and the packaged phage are amplified by growing a plate stock. (This cloning technique is described generally in Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Publications, pp. 256-293.)

Approximately 5×10^5 plaque forming units (pfu) from this plate stock are then screened with radiolabeled p4.7R by hybridization and autoradiography. Plaques which show hybridization to the p4.7R probe are plaque-purified and rescreened according to the above procedure. Positive plaques from the rescreening are isolated and used to prepare DNA.

putatively containing Rb alleles from the individual.

The MboI genomic inserts in these isolated EMBL3 vector DNA samples are tested for the location of the sequences homologous to p4.7R by Southern analysis. DNA samples containing the entire Rb gene region are selected, and the appropriate restriction fragments containing the Rb gene from these samples are subcloned into a suitable vector, such as pUC9. These subclones thus contain copies of one or both Rb alleles from the DNA of the individual to be tested. To determine if both alleles are represented, the initial phage isolates are tested for the existence of restriction polymorphisms. These subcloned alleles are then examined for differences from p4.7R by one of the following techniques.

First, the nucleotide sequence of the normal Rb gene in p4.7R is determined. Restriction fragments of approximately 500 base pairs (bp) from p4.7R are subcloned into an M13mp8 phage vector and sequenced by the dideoxy technique (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA, Vol. 74, pp. 5463-). A composite sequence of the Rb gene then can be assembled from these individual subclone sequences. The complete sequence of the normal retinoblastoma gene and flanking sequences is shown in Fig. 5 and Fig. 6.

The isolated Rb gene alleles are sequenced according to the following procedure. Restriction fragments (~ 2kb) of the allele are subcloned into the M13mp8 vector, and short

stretches (~500 bp) are sequenced individually using small restriction fragments isolated from p4.7R as the primers in the dideoxy sequencing reactions. The composite nucleotide sequence of the isolated allele then can be constructed from these individually-primed sequences. This sequence is compared directly with the sequence of the normal Rb gene, determined from p4.7R, to reveal any deletions or point mutations in the isolated allele.

An alternative method of comparing the allelic DNA with the normal Rb gene employs RNase A to assist in the detection of differences between the p4.7R sequence and the allele sequence. This comparison is performed in steps using small (~500 bp) restriction fragments of p4.7R as the probe. First, p4.7R is digested with a restriction enzyme(s) that cuts the Rb gene sequence into fragments of approximately 500bp. These fragments are separated on an electrophoresis gel; purified from the gel and cloned individually, in both orientations, into an SP6 vector (e.g., pSP64 or pSP65; Melton et al., 1984, Nucleic Acids Res., Vol. 12, pp. 7035-). The SP6-based plasmids containing inserts of p4.7R fragments are transcribed in vitro using the SP6 transcription system, well known in the art, in the presence of [α - 32 P]GTP, generating radiolabeled RNA transcripts of both strands of the cDNA of the Rb gene.

Individually, these RNA transcripts are used to form

heteroduplexes with the allelic DNA, as described by Myers et al., 1985, Science, Vol. 230, pp. 1242-46, the teachings of which are incorporated herein by reference. Mismatches that occur in the RNA:DNA heteroduplex, owing to sequence differences between the p4.7R fragment and the Rb allele subclone from the individual, result in cleavage in the RNA strand when treated with RNase A. Such mismatches can be the result of point mutations or small deletions in the individual's Rb allele. Cleavage of the RNA strand yields two or more small RNA fragments, which run faster on the denaturing gel than the RNA probe itself.

In the RNase A technique, radiolabeled Rb gene RNA is hybridized to single strands of an individual's Rb alleles which have been cloned into a vector. The RNase A technique is advantageous, however, because it also can be used without having to clone the Rb alleles. Preferably, genomic DNA is isolated from blood cells of the individual to be tested, and this genomic DNA is hybridized directly with the radiolabeled Rb RNA probes to determine sequence differences from the normal Rb gene. Specifically, 5 µg of isolated, total genomic DNA is resuspended with the labeled RNA probe in 30 µl of hybridization buffer (80% formamide, 40mM Pipes pH6.4, 0.4M NaCl, and 1mM EDTA), and this hybridization mix is treated at 90°C for 10 minutes to denature the DNA. The mixture then is cooled slowly to 45°C and incubated at this temperature for 10

hours to allow hybridization of the RNA probe to the single-stranded DNA copies of the Rb allele. After hybridization, RNase A treatment and electrophoresis are performed as described by Myers et al., supra. Mismatches between the RNA probe and the genomic copies of the individual's Rb alleles are then readily detected.

Detection of RFLPs Linked to the Rb Gene

The inheritance of a retinoblastoma- predisposing defect can be traced by following its co-inheritance with DNA polymorphisms in a pedigree analysis.

The gene map shown in Figure 2 was used to develop nucleic acid probes useful for retinoblastoma diagnosis. To do so, the bacteriophage DNA corresponding to the human inserts were subcloned in the plasmid vector "Bluescribe" (Stratagene). Fifteen single-copy DNA fragments from the gene, ranging in size from 500 bp to 2000 bp, were subcloned. These sequences are scattered over the 200 kb of the mapped region. Subcloned DNA fragments were separated from vector sequences by digestion of plasmid DNA with one or more restriction endonucleases, electrophoresis through a 0.6% low-melting-point agarose gel, and purification by chromatography using an Elutip-d column (Schleicher and Schuell). Purified DNA fragments were radiolabeled with ^{32}P -dCTP (New England Nuclear) by the random primer technique using the Klenow fragment of DNA polymerase I.

Restriction fragment length polymorphisms (RFLP's) were discovered by digesting genomic DNA isolated from six normal individuals with 33 different restriction enzymes. The DNA fragments resulting from the 198 separate digests were separated on a 0.8% agarose electrophoresis gel according to molecular shape or molecular weight. The DNA was transferred to nitrocellulose filters and hybridized with single copy DNA probes purified from the retinoblastoma gene according to published methods (T.P. Dryja et al., 1986, Proc. Natl. Acad. Sci. USA, Vol. 83, pp. 7391-94).

Of the 15 single-copy probe fragments, only five reveal RFLP's. Four of the polymorphisms appear to be the result of minor alterations (perhaps single base changes) in the recognition sequence of a restriction endonuclease (KpnI, XbaI, MboII, or Tth111I). The fifth polymorphism reflects variability in the number of tandem repeats of a 50 base pair sequence. The location of the DNA polymorphisms are shown in the map in Figure 2 (vertical arrows above the map). The location of the polymorphic MboII site(s) has not been determined precisely but is located at approximately 175 kb on this map. The frequencies of alleles which correspond to particular DNA polymorphisms are indicated in Table 1.

In order to demonstrate the utility of these probes to detect the presence of retinoblastoma-predisposing alleles in humans, twenty pedigrees with hereditary retinoblastoma were

analyzed. DNA was extracted from leucocyte nuclei of venous blood from available family members according to the method of Kunkel et al., 1977, Proc. Natl. Acad. Sci. USA, Vol. 74, pp. 1245-49, hereby incorporated by reference. For analysis of a kindred with a given RFLP, DNA from the available family members was digested with the appropriate restriction endonuclease. The resulting fragments were separated by agarose-gel electrophoresis, transferred to nitrocellulose filters, and hybridized to labeled probe.

In these families, the inheritance of alleles determined by the DNA polymorphisms within the retinoblastoma gene were traced and compared to the inheritance of the retinoblastoma-predisposing trait. For example, consider the polymorphism detected by probe p68RS2.0 (see Table 1). When genomic DNA is digested with the restriction enzyme RsaI, this probe hybridizes to allelic DNA fragments of different lengths. The size of these fragments ranges from 1.5 kb to 2.0 kb with intervals of approximately 50 bp. The DNA sequence of the 2.0 kb genomic fragment cloned in p68RS2.0 has a 50 to 53 bp segment which is repeated approximately 30 times (Table 2). This 53bp segment can be used as a probe in these analyses. A portion of the repeated sequence has homology to core sequences of VNTR's (Variable Number of Tandem Repeat) reported elsewhere (Y. Nakamura et al., 1987, Science, Vol. 235, pp. 1616-22). (The 11 bp sequence shown in Table 2 above the repeat unit

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TABLE 1
DNA POLYMORPHISMS
IDENTIFIED WITHIN THE RETINOBLASTOMA GENE

DNA PROBE	RESTRICTION ENDONUCLEASE	MAP LOCATION ¹	ALLELES SIZE (Kb) ³	FREQUENCY ⁴
p68RS2.0	<u>Rsa</u> I	142-143 kb	2.00	0.13
			1.95	0.02
			1.90	0.07
			1.85	0.07
			1.80	0.35
			1.75	0.20
			1.65	0.09
			1.50	0.09
p88PR0.6 ⁵	<u>Xba</u> I	120 kb	7.0	0.55
			5.5	0.45
p35R0.6	<u>Tth</u> 1111I	195 kb	4.95	0.20
			4.35	0.80
p2P0.3	<u>Mbo</u> II	175 kb ²	1.0	>.90
			0.8	<.05
			0.6	<.05
			0.3	<.05
p95HS0.5	<u>Kpn</u> I	25 kb	12.0	0.95
			8.0	0.05

¹The map location of each polymorphic site refers to the position on the restriction map of the gene shown in Figure 1.

²The location of the MboII site is approximate, since the precise position of this site within the map is not yet known.

³The allele sizes were calculated from several independent measurements using HindIII fragments of lambda phage DNA as a standard.

⁴Allele frequencies are based on a population of 40-60 unrelated individuals.

⁵p88PRO.6, p35RO.6, p2PO.3 and p95HS0.5 are probes isolated from other regions of the retinoblastoma gene, shown in Fig. 2.

represents the core sequence reported for some VNTR's observed by Nakamura et al.) Because such tandemly repeated sequences tend to be genetically unstable, the number of repeats is highly variable. Eight distinct alleles at this site have been detected, and more may exist. Because of this number of common alleles, seventy-five percent of unrelated individuals are heterozygous for this polymorphism. The high frequency of heterozygosity makes this polymorphism extremely useful. (In Table 2, the brackets "(" and ")" denote regions of variability within the repeat unit; and the bases underlined above and below the bracketed regions denote possible alternate bases for those regions of variability.)

TABLE 2
SEQUENCE OF THE REPEAT UNIT WITHIN p68RS2.0

	<u>TG</u>	<u>A</u>		GGGNNGTGGGG		<u>AG</u>
CGTGTGAA(<u>A</u>	<u>C</u>	TCCCCAATGCTGGAGGTGAGGTTTGGT(<u>AGAAG</u>)	ATGACT
	<u>G</u>					

Fourteen retinoblastoma families carried constellations of alleles at this DNA polymorphism. This variation allows an examination of the frequency of co-inheritance of this site with the retinoblastoma-predisposing trait. For example, genomic DNA of individuals in these families was digested with Rsa I and the co-inheritance of any one Rsa I fragment with defective Rb alleles determined.

Sequence Analysis

RFLP analysis can reveal only those sequence variations that give rise to a restriction fragment that is detectably different on a Southern blot from restriction fragments characterizing the normal nucleic acid. Most such detectable polymorphisms result from DNA sequence variation within a restriction endonuclease recognition site. These sites are rare, and finding them requires a laborious and expensive screening process in which genomic DNAs from several unrelated individuals are digested with as many as 50 different restriction enzymes. The fraction of all genomic DNA sequence polymorphisms (DSPs) at a specific locus that can be detected as RFLPs is small and depends on the number of enzymes used for screening; generally 90% or more of the DNA sequence polymorphism in the human genome is not within reach of RFLP-based analysis. Tens or even hundreds of potentially useful DSPs may exist within or near most disease-causing genes, but often only a few and sometimes none of these DSPs are detectable as RFLPs. Fig. 4 shows a scale map of the 200 kilobase genomic region that includes the 27 exons of the human retinoblastoma gene. The 27 exons make up a 4.7 kilobase transcript.

Fig. 7 shows the locations of the DNA sequence polymorphisms (DSPs) identified in this gene. Polymorphisms identified by the name of a restriction enzyme are RFLPs;

polymorphisms RB1.2, RB1.3, RB1.20 and RB1.26 are not detectable as RFLPs, and were found by PCR-amplification and direct sequencing, as described below. The 200 kilobase genomic region was isolated in a series of overlapping inserts from 35 distinct recombinant bacteriophage lambda clones. Exon-containing segments were subcloned into bluescribe plasmid cloning vectors (Stratagene, Inc.). Initial sequencing of cloned plasmid inserts was carried out using conventional methods for plasmid sequencing. Based on this genomic sequence, pairs of 20-base oligonucleotide primers were synthesized so that numerous regions 320 - 1200 bp in size could be amplified from genomic DNA by the polymerase chain reaction (PCR) method of Mullis et al.

For each amplification reaction, from 200 ng to 1.0 ug of genomic DNA was prepared in a reaction buffer containing 20 mM Tris (pH 8.4 or pH 8.6), 30 ug/ml bovine serum albumin, 300 mM to 7.5 mM, 10-50 pM of each oligonucleotide primer, and 1 unit of Taq polymerase (Perkin-Elmer Cetus). Optimal MgCl₂ concentrations and pH of the PCR reactions varied depending on the primer pair. PCR-amplification (30-35 rounds) was carried out following a cycle of 10 seconds at 94°C (denaturation), 10 seconds at 42-55°C (annealing), and 30 seconds at 70°C (Polymerization) using a programmable thermal cycler (Ericomp Corp., San Diego). All times are based on sample temperature rather than heat-block temperature, and do not include 'ramping

time' for the heat block. Optimal annealing temperatures varied for each primer pair. This protocol allowed PCR-amplification of regions as large as 2500 bp. Genomic DNA from 9-20 individuals was amplified for each region and screened for DSP.

In order to detect and utilize a greater fraction of the existing DNA sequence polymorphism in the retinoblastoma gene, we applied techniques of polymerase chain reaction (PCR), generally as described by K.B. Mullis et al., 1987, Methods Enzymol., Vol. 155, pp. 335-51, and direct sequencing, generally as described by C. Wong et al., 1987, Nature, Vol. 330, 384-86, to analyse normal allelic variation at this locus. Oligonucleotide primers were synthesized to amplify regions from the gene that varied in size from 320 - 1200 bp. Amplification and sequencing were carried out on DNA from at least 9 unrelated individuals for all regions screened, though for many regions 15 or more individuals were analyzed. In most cases, primer pairs were derived from intronic sequences that flanked one of the 27 exons of the gene, such that the PCR-amplified region contained both intron and exon sequences.

The results of this screening process are shown in Tables 3 and 4. Amplified DNA sequences were compared to one another and checked against sequence data from previously cloned plasmid inserts derived from the same region. Bases obscured by technical artifacts or other ambiguities were not

tabulated. Of 3712 bp of genomic DNA sequence screened at this locus, four sequence variations were identified (Table 3; map locations are shown in Fig. 7). All four variations were found in introns; of these, one is likely a rare variant (found in only 1 or 15 individuals sequenced), and three represent bona fide DNA sequence polymorphisms. A representative example (RB1.3) is illustrated in Fig. 7. This polymorphism occurs near exon 3 of the retinoblastoma gene. Neither form of the polymorphic sequence forms the recognition site of a known restriction enzyme, and hence this DSP is not detectable as an RFLP. Among a total of 82 genetically distinct (from unrelated individuals) alleles examined, no other base was observed at this site.

Fig. 8 illustrates the inheritance of the polymorphism RB1.3 in a retinoblastoma-prone family. Oligonucleotide primers (see Table 4) were used to PCR-amplify a 530 bp region of the human retinoblastoma gene that includes exon 3. The amplified fragment was sequenced by the methods described below. The sequence surrounding the polymorphism is written at the left side of the figure, read 5' to 3' from bottom to top, and the polymorphic bases are identified by adjacent tic marks.

The details of the analysis were as follows. Prior to sequencing, all PCR-amplified DNA samples were treated with proteinase-K and extracted with phenol/chloroform. High molecular weight DNA was separated from unused dNTPs and

oligonucleotide primers by column purification through sepharose CL-6B (Pharmacia). 250-400 ng of double-stranded PCR-amplified template was combined with 1-2 pM of (32 P) end-labeled sequencing primer, and heat-denatured for 3 minutes at 96°C. This primer-template mixture was added to a buffer containing: MgCl₂ (2.5 mM), Tris-HCL pH 7.5 (5 mM), 6 units Sequenase (U.S. Biochemical) and dithiothreitol (3 mM), and divided into 4 reaction mixtures each containing all four deoxynucleotides (32 uM each) and one dideoxynucleotide (5 uM). This mixture was immediately incubated for 5 minutes at 37-42°C, and polymerization was stopped with a 0.37% EDTA stop buffer. Prior to loading on sequencing gels, the samples were heat denatured at 96°C for 2 minutes. Conventional 0.4 mm thick, 6% polyacrylamide sequencing gels were used, and autoradiography was typically for 12-24 hours without an intensifying screen.

Fig. 9 illustrates segregation of the DSP RB1.3 in three families with hereditary retinoblastoma. Alleles are shown beneath the symbol for each person. Affected individuals are indicated by filled symbols. In family RB-32, the (-) allele is the result of an intragenic deletion. By subsequent Southern blotting studies, the deletion was found to extend from exon 2 to exon 17. Based on these results, it can be predicted that the unaffected members of family RB-32 who carry the (G,-) genotype are also carriers of the mutation.

The DSPs we have detected are valuable genetic markers for our studies of hereditary retinoblastoma. In its hereditary form, a predisposition to the disease is passed from affected individuals to their offspring as a dominant trait with 90% penetrance. It can be seen from Fig. 8 that the affected father, who has passed the disease to two children, is heterozygous for RB1.3. Both affected children received the G allele, while the unaffected child inherited the allele marked by an A at this polymorphic site. In this family, then, inheritance of the G allele from the affected parent is in phase with and diagnostic for the disease-predisposing phenotype. Fig. 9 shows our analysis of three other retinoblastoma-prone families using RB1.3. Inheritance of the polymorphic markers we describe here has followed the expected Mendelian pattern in every family examined so far. No cross-overs were observed between the polymorphic sites and the retinoblastoma-predisposing trait in any of the pedigrees. This follows our expectations since the polymorphisms are within the disease gene. In family RB-32, an intragenic deletion in one copy of the RB gene, presumably causing the predisposition to the tumor, was identified by Southern blotting (data not shown). The deletion includes the region surrounding RB1.3, and hence carriers of the disease-predisposing allele are genotypically hemizygous for the A allele (A,-). Two unaffected members of pedigree RB-32

are carriers for the disease-predisposition, based on analysis of RB1.3 (see Fig. 9). More happily, Fig. 9 shows that the other unaffected children in pedigree RB-32, as well as those in pedigrees RB-36 and RB-50, are not carriers of the cancer-predisposition and therefore will not pass the disease on to their children. These results highlight the diagnostic value of this class of human genetic markers that were heretofore unavailable for this purpose.

The data we present may also be used to estimate the level of heterozygosity in the human genome from a novel perspective. Previous estimates based on restriction enzyme screening may be subject to a bias because the sequences recognized by these enzymes do not necessarily reflect a random sampling. It is likely that a substantially higher level of polymorphism occurs at CpG pairs than elsewhere. This is reflected by the relatively high proportion of RFLPs revealed by such enzymes as Msp I (CCGG) and Taq I (TCGA). The method we describe is not subject to this bias. From the results of our screening, it can be calculated that genomic heterozygosity at this locus is approximately $h=0.00039$. If only the intron sequences are considered, this estimate increases to $h=0.00070$. These estimates are below the predictions of others, and may reflect the absence from our methods of the bias described above. However, an analogous calculation of heterozygosity ($0.00044 < h < 0.00087$) based instead on our

initial RFLP screening is also below the estimates of others and is quite consistent with our estimate based on direct sequencing. It seems likely that the human retinoblastoma gene is intrinsically less polymorphic than many other regions of the genome. Although mutations in this gene are known to be early events in the formation of several types of cancer, it is unclear why polymorphism at this locus may have been selected against in human evolution.

The approach for detecting DSPs demonstrated here has several advantages over conventional RFLP-based screening. As we have argued, DSP screening by amplification and direct sequencing could increase by an order of magnitude the number of available polymorphic markers at any cloned locus. This technique encompasses and supercedes restriction enzyme-based screening since RFLPs and VNTRs may also be detected. As the only requirement for utilization of such markers is knowledge of a unique set of amplification primer sequences and of the polymorphism itself, publication of a polymorphism immediately makes it available to all readers. Hence, problems and delays associated with the physical transfer of plasmid DNAs between laboratories are avoided, and the costs of maintaining plasmid repositories will be ultimately reduced. In addition, rapid analysis of these polymorphic markers can be carried out on a large scale with the use of allele-specific oligonucleotide probes for direct hybridization to amplified DNA. Finally,

Treatment of Patients Having a Defective Rb Gene

To prevent the formation of retinoblastoma in these
als, the Rb polypeptide is administered therapeutically
ount sufficient to inhibit retinoblastoma tumor
n or growth (anti-retinoblastoma-forming amount). An
inoblastoma-forming dosage of the Rb polypeptide is 1
g/kilogram of body weight/day. The Rb protein can be
ered by injection with a pharmacologically acceptable
either alone or in combination with another agent.
le pharmacological carriers are those which dissolve
olypeptide or hold it in suspension, and which are not
the extent of permanently harming the patient.
d are aqueous solutions of salts or non-ionic compounds
sodium chloride or glucose, most preferably at an
e concentration. Other agents may be present provided
y do not interfere with the action of the Rb
ide. Those skilled in the art will know, or will be
ascertain with no more than routine experimentation,

particular pharmacological carriers for this composition.

Rb polypeptide suitable for therapy can be prepared by any one of the following three conventional procedures. First, the Rb polypeptide can be produced by cloning the Rb cDNA from p4.7R into an appropriate mammalian expression vector, expressing the Rb gene product from this vector in an in vitro expression system, and isolating the Rb polypeptide from the medium or cells of the expression system. General expression vectors and systems are well known in the art.

Second, the Rb polypeptide can be produced using protein chemistry techniques, wherein the specific amino acid residues are joined together synthetically in the appropriate sequence.

Third, naturally occurring Rb protein can be isolated from total protein samples by affinity chromatography. Antibodies specific for the Rb protein are prepared by standard procedures (see below) and coupled to an inert matrix, which is then used to selectively bind the Rb proteins.

Immunodiagnosis of Retinoblastoma

This invention also includes methods for determining whether a particular tumor is the result of an Rb gene abnormality. Since osteosarcomas and certain undifferentiated tumors can result from detectable lesions in the Rb gene, immunodiagnosis can be used to aid in the diagnosis of such tumors.

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In order to produce anti-Rb antibody, a rabbit is immunized with either naturally occurring Rb protein or Rb polypeptide produced as described above. The anti-Rb antibody generated is then labeled, e.g., radioactively, fluorescently, or with an enzyme such as alkaline phosphatase. The labeled antibody is used to determine whether human tumors are of defective Rb gene origin. This can be carried out using any conventional technique. For example, the tumor sample can be liquified and tested against the labeled antibody using a conventional ELISA (Enzyme-linked immunosorbent assay) format. Alternatively, human tissue samples (e.g., biopsy samples) can be tested for expression of the retinoblastoma protein by other immunological techniques, see e.g., I. Roitt, Interaction of Antigen and Antibody, In Essential Immunology, Fifth edition, Boston: Blackwell Scientific Publications, 1984, pp. 145-75.

Immune complexes will be detected in tumor samples which have antigens (e.g., retinoblastoma polypeptide) reactive with anti-Rb antibody. Tumors which lack these antigens presumptively have a defect (e.g., mutation or a deletion) in the retinoblastoma gene.

Deposits

Plasmids p2AR3.8 and p2AR0.9 were deposited on July 17, 1987 with the American Type Culture Collection, Rockville, Maryland, and assigned ATCC accession numbers 40,241 and 40,242, respectively.

The Applicants represent the ATCC is a depository affording permanence of the deposit and ready accessibility thereto by the public if a patent is granted. All restrictions on the availability to the public of the material so deposited will be irrevocably removed upon the granting of a patent. The material will be available during the pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 USC 122. The deposited material will be maintained with all the care necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposited microorganism, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of the patent, whichever period is longer. Applicant acknowledges its duty to replace the deposit should the depository be unable to furnish a sample when requested due to the condition of the deposit.

Other embodiments are within the following claims.

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Table 4. Polymorphic sequences characterized and primer pairs used for PCR-amplification

Polymorphic Sequence	Allele Frequency	Amplification Primer Pair	Fragment Size	Location
RB1.2: TAAATAAGATCTTAAG TAAATAAGA CTTAAG	>95% < 5%	5'-AAGTGTAAATGTTTCTTAAG-3' 5'-TAGCAGAGGTAATTTCTC-3'	431 bp	124 bp from 5' end of exon 2
RB1.3: CAGAATCGTTTCCTTT CAGAATTCATTTCCTTT	73% 27%	5'-TTCAAATATATCCATCAGA-3' 5'-GCTTACACATGAATAGTGAGAG-3'	530 bp	43 bp from 3' end of exon 3
RB1.20: GATTT(CIII) _n CCTTTT n=14-26	N/D	5'-AATTAAACAAGGTGTGTGG-3' 5'-CTTGTAAATATGCTCATAAT-3'	550 600 bp	54 bp from 3' end of exon 20
RB1.26: ATTTTAAATCTGCAGT ATTTTAAATCTGCAGT	85% 15%	5'-ATTCAGTGAAGATATCTAAT-3' 5'-TAGTTCCTCTTTGTAGTTCT-3'	683 bp	10 bp from 5' end of exon 26

Shown are the sequences and locations of the polymorphic sites and their immediate flanking regions. Also shown are the oligonucleotide primers used to amplify these sequences from human genomic DNA. 'Fragment size' refers to the PCR-amplified product. Allele frequencies are based on analysis of the following numbers of individuals (of mixed North American descent): (RB1.2)-15; (RB1.3)-41; (RB1.20)-14; (RB1.26)-27. Accurate allele frequencies for RB1.20 have not yet been determined, as numerous alleles were found.

Table 3

DNA sequence polymorphisms detected by direct sequencing

Base Pairs Screened		Polymorphisms
Introns	2072	4
Exons	1640	0
<hr/>		<hr/>
Totals	3712	4

DNA sequence polymorphisms found by direct sequencing of 13 separate PCR-amplified regions from the human retionoblastoma locus. DNA samples from a minimum of 9 unrelated individuals were examined for all bases screened. Bases that could not be scored unambiguously were excluded from this tabulation.

66T430"36T4360

Claims

1. Purified nucleic acid comprising a human retinoblastoma gene, or a fragment thereof comprising 15 or more bases, said nucleic acid being less than 100kb in size.
2. A vector comprising the nucleic acid of claim 1.
3. A cell transformed with DNA encoding retinoblastoma polypeptide or a fragment thereof.
4. The nucleic acid of claim 1, wherein said nucleic acid hybridizes specifically to said retinoblastoma gene under hybridizing conditions.
5. An isolated polypeptide encoded by the nucleic acid of claim 1.
6. An antibody produced to the polypeptide of claim 5.
7. An antibody produced to naturally occurring retinoblastoma polypeptide.
8. A method of detecting large deletions in the retinoblastoma gene of a human patient predisposing said patient to retinoblastoma, comprising the steps of:
hybridizing a nucleic acid sample from said patient with a probe specific for the retinoblastoma gene, and
determining the ability of said probe to hybridize to said nucleic acid,
wherein lack of hybridization to said nucleic acid indicates the presence of a large deletion in said gene.
9. A method of detecting large deletions in the retinoblastoma gene of a human patient that may predisposing said patient to retinoblastoma, comprising the steps of:

generating nucleic acid fragments from a sample of said patient,

separating said fragments according to a determined physical property of said fragments,

hybridizing a probe specific for the retinoblastoma gene to said fragments,

detecting hybrids of said probe and said fragments, and comparing said hybrids to hybrids detected from the hybridization of said probe and separated nucleic acid fragments from a normal retinoblastoma gene,

wherein the absence of hybrids, or the smaller size of said hybrids from the sample of said patient is an indication of large deletions in the retinoblastoma gene of said patient.

10. The method of claim 8 or 9, wherein the probe specific for the retinoblastoma gene is the cloned DNA in p4.7R, or a fragment thereof.

11. The method of claim 9, wherein the physical property is molecular weight.

12. A method of detecting small deletions or point mutations in the retinoblastoma gene of a human patient predisposing said patient to retinoblastoma, comprising the steps of:

determining the nucleotide sequence of a retinoblastoma allele, or subregion thereof, from said patient, and

comparing said nucleotide sequence with the nucleotide sequence of a retinoblastoma allele or region thereof from a person not afflicted with retinoblastoma.

13. A method of detecting small deletions or point mutations in the retinoblastoma gene of a human patient predisposing said patient to retinoblastoma, comprising detecting mismatches between a nucleic acid sample from said patient and a detectable probe specific for the retinoblastoma gene from a person not afflicted with retinoblastoma, wherein mismatches are an indication of small deletions or mutations in the retinoblastoma gene of said patient.

14. A method of diagnosing predisposition of a human patient to retinoblastoma, comprising detecting the co-inheritance of retinoblastoma alleles of said patient with DNA polymorphisms in a pedigree analysis.

15. A method of detecting genetic polymorphisms, in the retinoblastoma gene of a human patient, that predispose said patient to retinoblastoma, comprising the steps of:

generating nucleic acid fragments from a sample of said patient,

separating said fragments according to a determined physical property of said fragments,

hybridizing a detectable nucleic acid probe capable of hybridizing to the wild type retinoblastoma gene to said fragments,

detecting hybrids of said probe and said fragments, and

comparing said hybrids to hybrids detected from the hybridization of said probe and separated nucleic acid fragments from a sample of a parent of said patient,

wherein the co-inheritance of specific genetic polymorphisms with the retinoblastoma gene is an indication of the predisposition of said patient to retinoblastoma.

16. The method of claim 15, wherein said physical property is molecular weight.

17. A method of treating a human patient having a defective retinoblastoma gene comprising administering to said patient an anti-retinoblastoma-forming amount of the retinoblastoma polypeptide.

18. A composition suitable for treating a human patient having a defective retinoblastoma gene, comprising retinoblastoma polypeptide and a pharmacologically acceptable carrier therefor.

19. A method of detecting the presence, in a tumor sample, of a protein the absence of which is associated with a neoplasm, said method comprising producing an antibody to said protein, contacting said antibody with said tumor sample, and detecting immune complexes as an indication of the presence in said tumor sample of said protein.

20. A method of detecting the presence of the retinoblastoma protein in a tumor sample from a human patient, comprising the steps of:

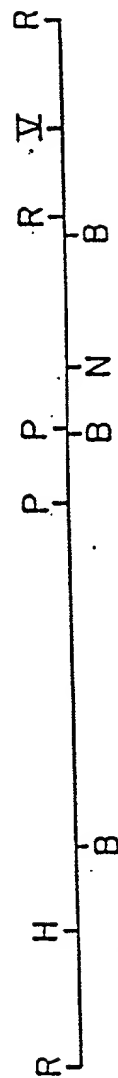
contacting said tumor sample with an antibody which specifically reacts with the retinoblastoma protein, and

determining whether immune complexes are formed with said antibody, the formation of said immune complexes being an indication that the tumor is not retinoblastoma and the absence of immune complexes indicating that the tumor is retinoblastoma.

21. The method of claim 20, wherein said antibody is a monoclonal antibody.

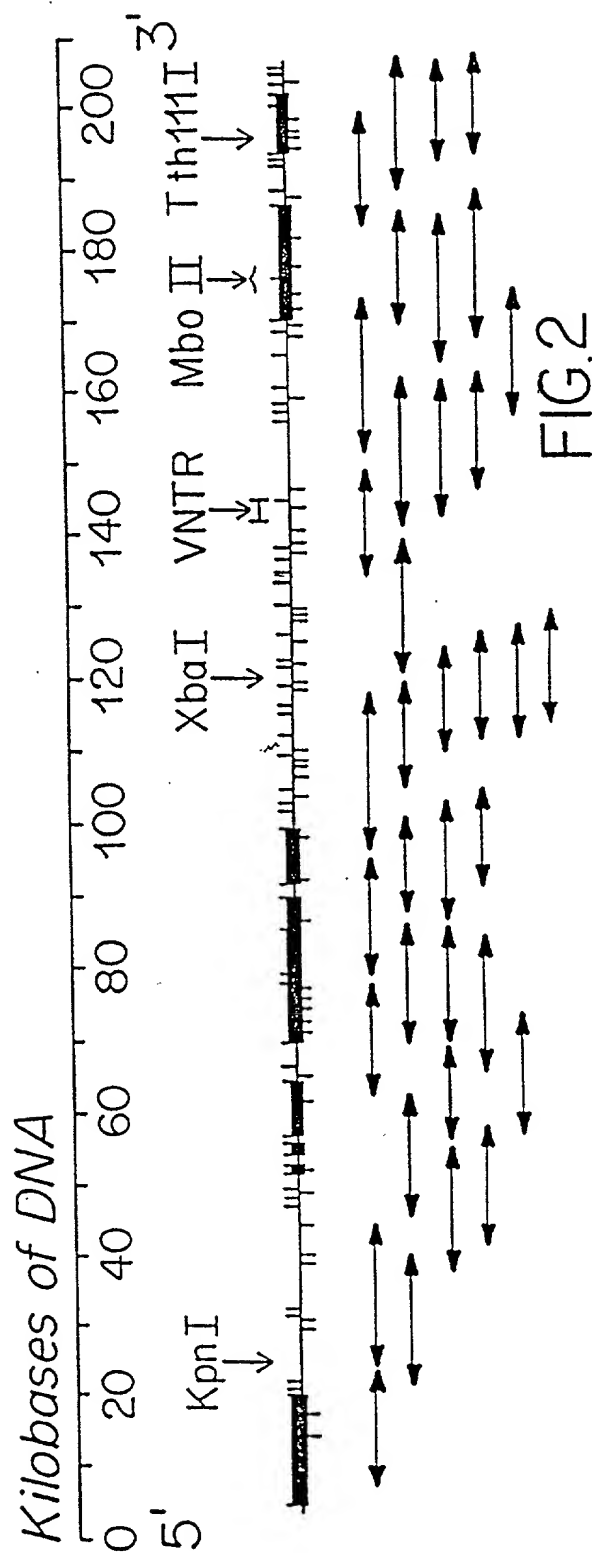
	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370	2371	2372	2373	2374	2375	2376	2377	2378	2379	2380	2381	2382	2383	2384	2385	2386	2387	2388	2389	2390	2391	2392	2393	2394	2395	2396	2397	2398	2399	2400	2401	2402	2403	2404	2405	2406	2407	2408	2409	2410	2411	2412	2413	2414	2415	2416	2417	2418	2419	2420	2421	2422	2423	2424	2425	2426	2427	2428	2429	2430	2431	2432	2
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1



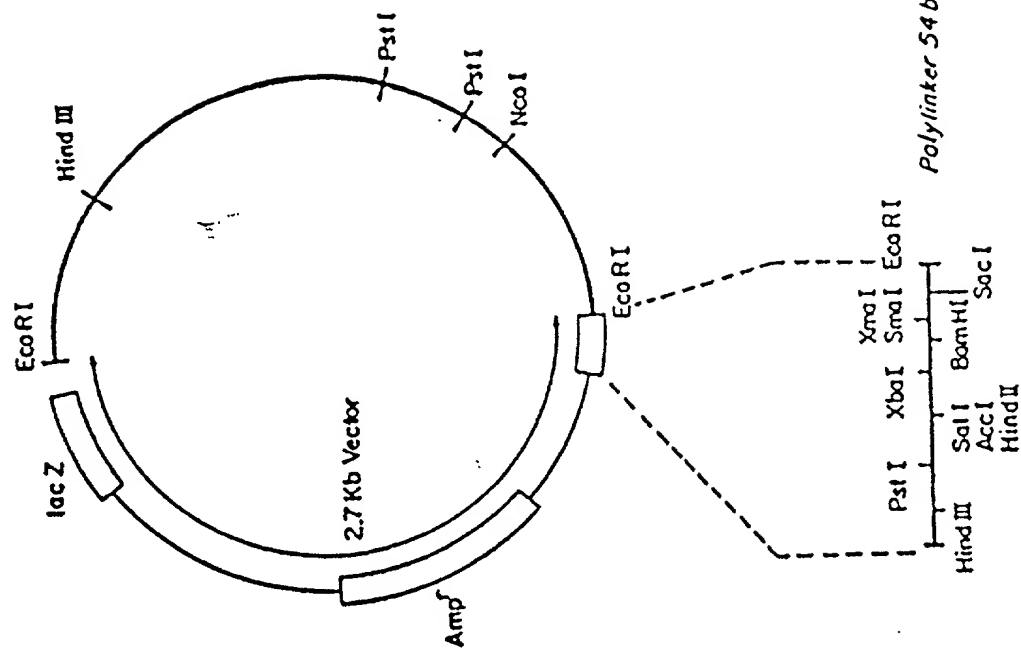
R \equiv EcoRI P \equiv Pst I
H \equiv Hind III N \equiv Nco I
B \equiv Bgl II V \equiv Eco RV

FIG.1



p2AR38

p3.8R in pUC13



p2AR0.9

p0.9R in pSP65

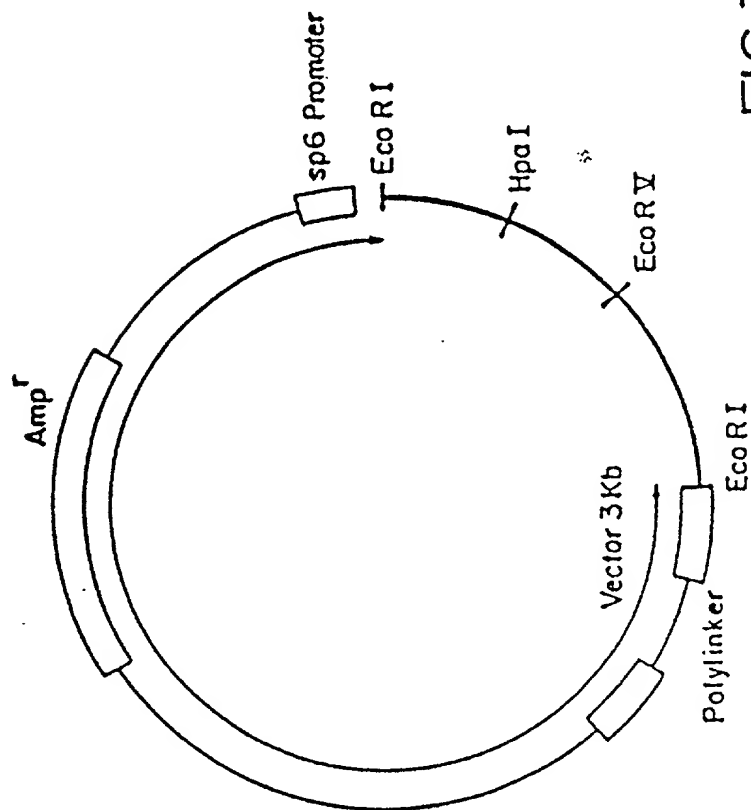
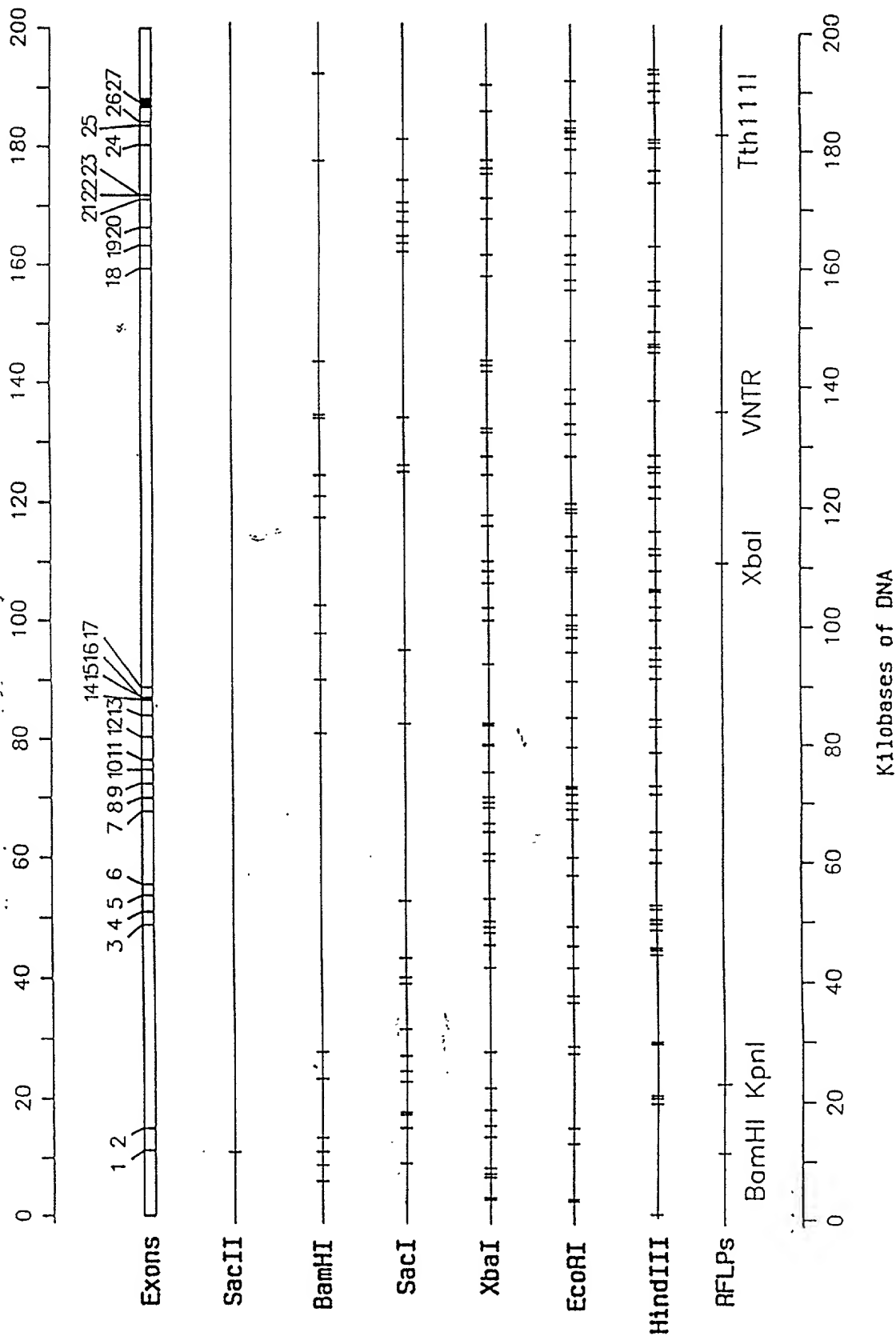


FIG.3

F-g. 4

MAP OF THE RETINOBLASTOMA GENE



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100

337	ATG M	TCG S	TTC F	ACT T	TTT F	ACT T	GAG E	CTA L	CAG Q	AAA K	AAC N	ATA I	GAA E	ATC I	378
379	AGT S	GTC V	CAT H	AAA K	TTC F	TTT F	AAC N	TTA L	CTA L	AAA K	GAA E	ATT I	GAT D	ACC T	420
421	AGT S	ACC T	AAA K	GTT V	GAT D	AAT N	GCT A	ATG M	TCA S	AGA R	CTG L	TTG L	AAG K	AAG K	462
463	TAT Y	GAT D	GTA V	TTG L	TTT F	GCA A	CTC L	TTC F	AGC S	AAA K	TTG L	GAA E	AGG R	ACA T	504
505	TGT C	GAA E	CTT L	ATA I	TAT Y	TTG L	ACA T	CAA Q	CCC P	AGC S	AGT S	TCG S	ATA I	TCT S	546
547	ACT T	GAA E	ATA I	AAT N	TCT S	GCA A	TTG L	GTG V	CTA L	AAA K	GTT V	TCT S	TGG W	ATC I	588
589	ACA T	TTT F	TTA L	TTA L	GCT A	AAA K	GGG G	GAA E	GTA V	TTA L	CAA Q	ATG M	GAA E	GAT D	630
631	GAT D	CTG L	GTG V	ATT I	TCA S	TTT F	CAG Q	TTA L	ATG M	CTA L	TGT C	GTC V	CTT L	GAC D	672
673	TAT Y	TTT F	ATT I	AAA K	CTC L	TCA S	CCT P	CCC P	ATG M	TTG L	CTC L	AAA K	GAA E	CCA P	714
715	TAT Y	AAA K	ACA T	GCT A	GTT V	ATA I	CCC P	ATT I	AAT N	GGT G	TCA S	CCT P	CGA R	ACA T	756
757	CCC P	AGG R	CGA R	GGT G	CAG Q	AAC N	AGG R	AGT S	GCA A	CGG R	ATA I	GCA A	AAA K	CAA Q	798
799	CTA L	GAA E	AAT N	GAT D	ACA T	AGA R	ATT I	ATT I	GAA E	GTT V	CTC L	TGT C	AAA K	GAA E	840
841	CAT H	GAA E	TGT C	AAT N	ATA I	GAT D	GAG E	GTG V	AAA K	AAT N	GTT V	TAT Y	TTC F	AAA K	882
883	AAT N	TTT F	ATA I	CCT P	TTT F	ATG M	AAT N	TCT S	CTT L	GGA G	CTT L	GTA V	ACA T	TCT S	924
925	AAT N	GGA G	CTT L	CCA P	GAG E	GTT V	GAA E	AAT N	CTT L	TCT S	AAA K	CGA R	TAC Y	GAA E	966
967	GAA E	ATT I	TAT Y	CTT L	AAA K	AAT N	AAA K	GAT D	CTA L	GAT D	GCA A	AGA R	TTA L	TTT F	1008
1009	TTG L	GAT D	CAT H	GAT D	AAA K	ACT T	CTT L	CAG Q	ACT T	GAT D	TCT S	ATA I	GAC D	AGT S	1050
1051	TTT F	GAA E	ACA T	CAG Q	AGA R	ACA T	CCA P	CGA R	AAA K	AGT S	AAC N	CTT L	GAT D	GAA E	1092
1093	GAG E	GTG V	AAT N	GTA V	ATT I	CCT P	CCA P	CAC H	ACT T	CCA P	GTT V	AGG R	ACT T	GTT V	1134
1135	ATG M	AAC N	ACT T	ATC I	CAA Q	CAA Q	TTA L	ATG M	ATG M	ATT I	TTA L	AAT N	TCA S	GCA A	1176
1177	AGT S	GAT D	CAA Q	CCT P	TCA S	GAA E	AAT N	CTG L	ATT I	TCC S	TAT Y	TTT F	AAC N	AAC N	1218

Fig. 5-2

1219	TGC ACA GTG AAT CCA AAA GAA AGT ATA CTG AAA AGA GTG AAG	1260
	C T V N P K E S I L K R V K	
1261	GAT ATA GGA TAC ATC TTT AAA GAG AAA TTT GCT AAA GCT GTG	1302
	D I G Y I F K E K F A K A V	
1303	GGA CAG GGT TGT GTC GAA ATT GGA TCA CAG CGA TAC AAA CTT	1344
	G Q G C V E I G S Q R Y K L	
1345	GGA GTT CGC TTG TAT TAC CGA GTA ATG GAA TCC ATG CTT AAA	1386
	G V R L Y Y R V M E S M L K	
1387	TCA GAA GAA GAA CGA TTA TCC ATT CAA AAT TTT AGC AAA CTT	1428
	S E E E R L S I Q N F S K L	
1429	CTG AAT GAC AAC ATT TTT CAT ATG TCT TTA TTG GCG TGC GCT	1470
	L N D N I F H M S L L A C A	
1471	CTT GAG GTT GTA ATG GCC ACA TAT AGC AGA AGT ACA TCT CAG	1512
	L E V V M A T Y S R S T S Q	
1513	AAT CTT GAT TCT GGA ACA GAT TTG TCT TTC CCA TGG ATT CTG	1554
	N L D S G T D L S F p W I L	
1555	AAT GTG CTT AAT TTA AAA GCC TTT GAT TTT TAC AAA GTG ATC	1596
	N V L N L K A F D F Y K V I	
1597	GAA AGT TTT ATC AAA GCA GAA GGC AAC TTG ACA AGA GAA ATG	1638
	E S F I K A E G N L T R E M	
1639	ATA AAA CAT TTA GAA CGA TGT GAA CAT CGA ATC ATG GAA TCC	1680
	I K H L E R L E H R I M E S	
1681	CTT GCA TGG CTC TCA GAT TCA CCT TTA TTT GAT CTT ATT AAA	1722
	L A W L S D S P L F D L I K	
1723	CAA TCA AAG GAC CGA GAA GGA CCA ACT GAT CAC CTT GAA TCT	1764
	Q S K D R E G P T D H L E S	
1765	GCT TGT CCT CTT AAT CTT CCT CTC CAG AAT AAT CAC ACT GCA	1806
	A C P L N L P L Q N N H T A	
1807	GCA GAT ATG TAT CTT TCT CCT GTA AGA TCT CCA AAG AAA AAA	1848
	A D M Y L S P V R S P K K K	
1849	GGT TCA ACT ACG CGT GTA AAT TCT ACT GCA AAT GCA GAG ACA	1890
	G S T T R V N S T A N A E T	
1891	CAA GCA ACC TCA GCC TTC CAG ACC CAG AAG CCA TTG AAA TCT	1932
	Q A T S A F Q T Q K P L K S	
1933	ACC TCT CTT TCA CTG TTT TAT AAA AAA GTG TAT CGG CTA GCC	1974
	T S L S L F Y K K V Y R L A	
1975	TAT CTC CGG CTA AAT ACA CTT TGT GAA CGC CTT CTG TCT GAG	2016
	Y L R L N T L C E R L L S E	
2017	CAC CCA GAA TTA GAA CAT ATC ATC TGG ACC CTT TTC CAG CAC	2058
	H P E L E H I I W T L F Q H	
2059	ACC CTG CAG AAT GAG TAT GAA CTC ATG AGA GAC AGG CAT TTG	2100
	T L Q N E Y E L M R D R H L	
2101	GAC CAA ATT ATG ATG TGT TCC ATG TAT GGC ATA TGC AAA GTG	2142
	D Q I M M C S M Y G I C K V	
2143	AAG AAT ATA GAC CTT AAA TTC AAA ATC ATT GTA ACA GCA TAC	2184
	K N I D L K F K I I V T A Y	

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Fig. 5-3

2185 AAG GAT CTT CCT CAT GCT GTT CAG GAG ACA TTC AAA CGT GTT 2226
 K D L P H A V Q E T F K R V

2227 TTG ATC AAA GAA GAG GAG TAT GAT TCT ATT ATA GTA TTC TAT 2268
 L I K E E E Y D S I I V F Y

2269 AAC TCG GTC TTC ATG CAG AGA CTG AAA ACA AAT ATT TTG CAG 2310
 N S V F M Q R L K T N I L Q

2311 TAT GCT TCC ACC AGG CCC CCT ACC TTG TCA CCA ATA CCT CAC 2352
 Y A S T R P P T L S P I P H

2353 ATT CCT CGA AGC CCT TAC AAG TTT CCT AGT TCA CCC TTA CGG 2394
 I P R S P Y K F P S S P L R

2395 ATT CCT GGA GGG AAC ATC TAT ATT TCA CCC CTG AAG AGT CCA 2436
 I P G G N I Y I S P L K S P

2437 TAT AAA ATT TCA GAA GGT CTG CCA ACA CCA ACA AAA ATG ACT 2478
 Y K I S E G L P T P T K M T

2479 CCA AGA TCA AGA ATC TTA GTA TCA ATT GGT GAA TCA TTC GGG 2520
 P R S R I L V S I G E S F G

2521 ACT TCT GAG AAG TTC CAG AAA ATA AAT CAG ATG GTA TGT AAC 2562
 T S E K F Q K I N Q M V C N

2563 AGC GAC CGT GTG CTC AAA AGA AGT GCT GAA GGA AGC AAC CCT 2604
 S D R V L K R S A E G S N P

2605 CCT AAA CCA CTG AAA AAA CTA CGC TTT CAT ATT GAA GGA TCA 2646
 P K P L K K L R F D I E G S

2647 GAT GAA GCA GAT GGA AGT AAA CAT CTC CCA GGA GAG TCC AAA 2688
 D E A D G S K H L P G E S K

2689 TTT CAG CAG AAA CTG GCA GAA ATG ACT TCT ACT CGA ACA CGA 2730
 F Q Q K L A E M T S T R T R

2731 ATG CAA AAG CAG AAA ATG AAT GAT AGC ATG GAT ACC TCA AAC 2772
 M Q K Q K M N D S M D T S N

2773 AAG GAA GAG AAA 2784
 K E E K

TGAGGATCTCAGGACCTTGGTGGACACTGTGTACACCTCTGGATTCAATTGTCTCTCACAGATGTGACTGTATAACTTTCC 2864
 CAGGTTCTGTTTATGGCCACATTTAATATCTTCAGCTCTTTTGTGGATATAAAATGTGCAGATGCAATTGTTTGGGTGA 2944
 TTCCTAAGCCACTTGAAATGTTAGTCATTGTTATTTATACAAGATTGAAATCTTTGTGTAATCCTGCCATTTAAAAAGT 3024
 TGTAGCAGATTGTTTCTCTTCCAAAGTAAATTTGCTGTGCTTTATGGATAGTAAGAATGGCCCTAGAGTGGGAGTCCTG 3104
 ATAACCCAGGCCTGTCTGACTACTTTGCCTTCTTTTGTAGCATATAGGTGATGTTTGCTCTTGTTTTATTAATTTATAT 3184
 GTATATTTTTTAAATTTAACATGAACACCCTTAGAAAATGTGTCCTATCTATCTTCCAAATGCAATTTGATTGACTGCC 3264
 ATTCACCAAAATTAATCCTGAACTCTTCTGCAAAATGGATATTATTAGAAATTTAGAAAAAATTTACTAATTTTACACATT 3344
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 AGCAAAGTATAACCATATGATACTATCATACTACTGAAACAGATTTTCATACCTCAGAATGTAAAAAGAACTTACTGATTAT 3504
 TTTCTTCATCCAACCTTATGTTTTTAAATGAGGATTATTGATAGTACTCTTGGTTTTATACCATTCAGATCACTGAATTT 3584
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 CAAGTGCACTTTCTAATGTTTCTGGGTCTGAAGAATTAAGATACAAATTAATTTTACTCCATAAACAGACTGTTAATTA 3744
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 TGTTAGAAAACAAAATTTTATTTTGTGCTCATTTAAGTTTCAAACTTACTATTTTGACAGTTATTTTGATAACAATGACA 4304
 CTAGAAAACTTGACTCCATTTTCATCTGTTTCTGCATGAATATCATACAAATCAGTTAGTTTGTAGGTCAAGGGCTTAC 4384
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 TTTCTTAAATAATGCTTCAGATATTATTGCTTTATTGCTTTTTTGTATTGGTTAAACTGTACATTTAAATTTGCTATGT 4544
 TACTATTTTCTACAATTAATAGTTTGTCTATTTTAAATAAATTAGTTGTTAG..... 4597

Fig. 6-1

<u>SacII</u> EXON 1	aaggaggag	agtggngtcc	ngnngagggt	gcactagcca	gatattctgc	ggggcccgag	060
	agtcttccct	atcagacccc	gggataggga	tgaggCCCAC	AGTCACCCAC	CAGACTCTTT	120
	GTATAGCCCC	GTTAAGTGCA	CCCCGGCCTG	GAGGGGGTGG	TTCTGGGTAG	AAGCACGTCC	180
	GGGCCGCGCC	GGATGCCTCC	TGGAAGGCGC	CTGGACCCAC	GCCAGGTTTC	CCAGTTTAAT	240
	TCCTCATGAC	TTAGNGTCCC	AGCCNGCGCA	CCJACCAGCG	CCCCAGTTCC	CCACAGACGC	300
	CGGCCGNNNC	GGGAGCCTGC	GGACGTGAGC	GCGGGCGGAA	GTGACGTTTT	CCGCGGTTGG	360
	ACGCGGCGCT	CAGTTGCCGG	GCGGGGGAGG	GCGCGTCCGG	TTTTTCTCAG	GGGACGTTGA	420
	start of cDNA sequence						
	AATTATTTTT	GTAACGGGAG	TGGGGAGAGG	ACGGGGCGTG	CCCCGCGTGC	GCGCGCGTCG	480
	TCCTCCCCGG	CGCTCCTCCA	CAGCTCGCTG	GCTCCCGCCG	CGGAAAGGCG	TCATGCCGCC	540
<u>SacII</u>	CAAAACCCCC	CGAAAAACGG	CGCCACCCGC	CGCCGCTGCC	GCCGCGGAAC	CCCCGGCACC	600
	oLysThrPro	ArgLysThrA	laAlaThrAl	aAlaAlaAla	AlaAlaGluP	roProAlaPr	(23)
	GCCGCGCGCG	CCCCCTCCTG	AGGAGGACCC	AGAGCAGGAC	AGCGGCCCGG	AGGACCTGCC	660
	oProProPro	ProProProG	luGluAspPr	oGluGlnAsp	SerGlyProG	luAspLeuPr	(43)
	TCTCGTCAGG	TGAGCGAGCA	GAGCGCGTCN	CTCACGCGGG	AAGGGCGCCC	CGGGTGTGCC	720
	oLeuValAr						(46)
	TAGGGCGGGC	GCAAGGCGgC	TGGGCGGGGA	CCCGTCCTCG	CCAGGGgCCG	GGTCCcgGNG	780
	GGAGGAGGCG	CCCTCCCTGC	CCCCCGCCAC	GGcggaGCGT	CTGCAGAATG	GTGACAGGAT	840
	TCTGGGTTCT	TGGGCGAGGG	GTCTCGGCTT	CAACTTGACA	GGTGTGGGGC	GGGTggggct	900
	agnntcctga	gcgaagtgc	aggtgcagtt	ccctcttggt	agnctcggan	ncagaggntc	960
EXON 2	gttgcgagcg	tncatcagac	aaaaaatga	aaataaaaa	tacaaaaa		1008
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	gatatttgac	ttaccatgca	agcaaatatt	tttactgtg	tggtatcctt	attttggaat	120
	gaccatgaaa	aagataatca	tatgnnnaaa	tttgaagtgt	aatgtttttc	taagataaaa	180
	taagatctta	AAGTATTTAA	TAATGTTCTT	TTTCACAGTA	GTGTTATGTG	CAAATATTG	240
	AAACAAGTAT	GTAATGAATC	AATTTGATTT	ATAAGATATG	CCAATTATAT	GATTATTTTC	300
	ATTTGGTAGG	CTTGAGTTTG	AAGAAACAGA	AGAACCTGAT	TTTACTGCAT	TATGTCAGAA	360
		g LeuGluPheG	luGluThrGl	uGluProAsp	PheActAlaL	euCysGlnLy	(63)
	ATTAAGATA	CCAGATCATG	TCAGAGAGAG	AGCTTGGTTA	ACTTGGGAGA	AAGTTTCATC	420
<u>SacI</u>	sLeuLysIle	ProAspHisV	alArgGluAr	gAleTrpLeu	ThrTrpGluL	ysValSerSe	(83)
	TGTGGATGGA	GTATTGGTAA	GGATTTTCTT	AAAACGTTTT	GAAATTTTTT	TTTCTCATTT	480
	rValAspGly	ValLeu					(88)
	TAAAACCAAC	TTCAATCAC	TATACAAAAA	TTGAAAGATA	GAAAAATATA	AAGACAATAA	540
	AAGctaataa	taattccatt	acccagagga	aatttacctc	tgctaacatt	aaaaatgttt	600
	gaggccgggc	acgtggttca	tgctgtaat	cctaccactt	tgggagsgctg	aggcagsgtg	660
	attgcctgag	ctcaggagtt	cgagaccagc	ctgggcaaca	tggt		704
	--33 kb--						
	ctatttgaga	tgactgaccc	ctaaagtctc	acaataacta	tttaattttt	tatctttcta	060
	atactttttt	gccttataat	ataaaatttg	aatgtttgtt	attagtgtga	aatgaaatcc	120
EXON 3	tttcaaata	atgccatcag	aaggatgtgt	tacaaatata	cagtATTACA	AACATTTATT	180
	TTGTATGCTG	AATAAGAAAA	AATCAGTTAT	AATACAGTTT	TAACATAGTA	TCCAGTGTGT	240
	GAATTATTTA	ATGAAATATT	TGATCTTTAT	TTTTTGTTC	AGGGAGGTTA	TATTCAAAAAG	300
					GlyGlyTy	rIleGlnLys	(94)
	AAAAAGGAAC	TGTGGGGAAT	CTGTATCTTT	ATTGCAGCAG	TTGACCTAGA	TGAGATGTCC	360
	LysLysGluL	euTrpGlyIl	eCysIlePhe	IleAlaAlaV	alAspLeuAs	pGluMetSer	(114)
	TTCACCTTTA	CTGAGCTACA	GAAAAACATA	GAAATCAGGT	AAAGTTTCTT	GTATAAATAT	420
	PheThrPheT	hrGluLeuGl	nLysAsnIle	GluIleSe			(127)
	AAGCCTCTGC	CATAAAAGGA	AACGAATTCT	GGATTTTCCT	CTCAATAGAC	TTTTGTGAAT	480
	TAGTGAGAAA	TGCTAAAATA	AAGTAAAACA	AAAAGAACTT	GGACCAATA	GTGAACTGCC	540
<u>EcoRI</u>	ATTCTCTCAT	GGAGCCGTTA	TGAAAGTGTA	TTTATGCTGT	ATTTCTTTAA	GAGGTAGCAG	600
	TTTGTGTCCT	GGAAAAATTT	TCATTGTGTC	TCTCACTATT	CATGTGTAAG	C	651
--1.6 kb--							

Fig. 6-2

EXON 4	gcataggtat	atagataata	gaggtgtaag	ttgaaggcta	attatTTTTg	caaaaagtaa	060
	ttccttccaa	aggatatagt	agtgatttga	tgtagagctg	ataatcTTTT	GAATTGAAAT	120
	ATCTATGATT	TGAAAACGAA	ATAACACAAA	TTTTTAAGGT	TACTGATTTA	CTTTTTTCTA	180
	TTCTTTTCCTT	TGTAGTGTCC	ATAAATTCTT	TAACCTACTA	AAAGAAATTG	ATACCAGTAC	240
		rValH	isLysPhePh	eAsnLeuLeu	LysGluIleA	spThrSerTh	(142)
	CAAAGTTGAT	AATGCTATGT	CAAGACTGTT	GAAGAAGTAT	GATGTATTGT	TTGCACTCTT	300
	rLysValAsp	AsnAlaMetS	erArgLeuLe	uLysLysTyr	AspValLeuP	heAlaLeuPh	(162)
	CAGCAAATTG	GAAAGGTAAA	GTAACATTTT	TATTAGGGTT	ACACTCTGAT	TTTTTATGTC	360
	eSerLysLeu	GluAr					(167)
	ATTGTTTACA	ATTAGATTCT	GGGAATTATT	TAACACATTT	AGTAAAGTTA	GTAAGTATTA	420
	ATTCTTAgac	ttgtcccttt	taatgttagc	tcattaattc	ttagctttct	tatttatcca	480
	gtaa ^a tatgca	ttctgaatgc	ttcctggaaa	attaaccgtt	attatccttt	catgtctcca	540
	tttgttttca	aaacttagct	tatcgagtat				570
	--2.1 kb--						
	gagatattta	aagagnaact	ttactaacct	taggtggatc	agctgggtgt	tttctatctt	060
	atttatacct	tttttttgaa	GACTAATTGA	GAGGATTAAC	TGTAATTATA	TATTAAAGTG	120
	ATGTGAGATG	TCATAAATTG	GGAAAATCTA	CTTGAACTTT	GTTTTATAAT	GCTATATATT	180
	TTTTGTTTTT	AAAATATATA	CTTCTTAAAA	GAAGATGAAT	AAAGCATGAG	AAAACtACTA	240
EXON 5	TGACTTCTAA	ATTACGAAAA	AATGTTAAAA	AGTCATAATG	TTTTTCTTTT	CAGGACATGT	300
					gThrCys		(169)
	GAACTTATAT	ATTTGACACA	ACCCAGCAGT	TCGTAAGTAG	TTCACAGAAT	GTTATTTTTT	360
	GluLeuIleT	yrLeuThrGl	nProSerSer	Se			(180)
	ACTTAAAAAA	AAAGATTTTT	ATGGAATAAT	CTCAAACATC	TTGATAGTTA	GGGTTAGTTT	420
	GATCGATTAT	AGCAGGCTAC	Ttcataaatt	aagcccatag	atttaagtcc	tgtgtagatt	480
	atttatcttc	tcacaaagaa	aatagtataa	aatacatgcc	ttgtactaca	aagaagaact	540
	aataaggtgg	aattgattca	ggacagcata	tcaccaactc	tgagaaaaat	gcaacaaatg	600
	caaattcatt	gactaa					616
	--1.4 kb--						
	aatggactg	cattctatta	tgcatttaac	taaggtcatt	ttttttttta	tGCACAAAAA	060
	GAAACACCCA	AAAGATATAT	CTGGAAAACT	TTCTTTCAGT	GATACATTTT	TCCTGTTTTT	120
	TTTCTGCTTT	CTATTGTGTT	AATAGGATAT	CTACTGAAAT	AAATTCTGCA	TTGGTGCTAA	180
		rIleS	erThrGluIl	eAsnSerAla	LeuValLeuL		(192)
	AAGTTTCTTG	GATCACAATT	TTATTAGCTA	AAGGTAAGTT	CATTATATTT	ATTAATGCT	240
	ysValSerTr	pIleThrPhe	LeuLeuAlaL	ysG			(203)
	AATATTTCAA	ATGTAATAAT	TAAATTGGCA	TTCCTTTGGG	CTAAATTCCT	CAATTTTTAT	300
	TGAGTAATGT	ACTCCTccct	cattctctgc	ttggcttatt	aactgttagc	aagttcctat	360
	aattctggta	ctagaaacaa	ccttggaaat	gctttattta	atntttgttt	ctaattattcc	420
	atcttccctc	cctt					434
	--11.5 kb--						
	tttatagtga	ttttagacat	aaagaattaa	ttataacaga	aatagcttaa	atgtaaaatt	060
	ctcagagtag	agcttaacac	ttgatttata	attccataac	tttcatattt	tCTATTTTAC	120
	ATATTTTATA	CCTTTTAAAA	CAGATTTTTT	TTTTTTTtAC	AAAAAAAAGA	AAGAAAATCT	180
	TTACCATGCT	GATAGTGATT	GTTGAATGAA	TAAATTTATG	GATATACTCT	ACCCTGCGAT	240
	TTTCTCTCAT	ACAAAGATCT	GAATCTCTAA	CTTCTTTTAA	AAATGTACAT	TTTTTTTTTCA	300
EXON 7	GGGGAAGTAT	TACAAATGGA	AGATGATCTG	GTGATTTTCA	TTCAGTTAAT	GCTATGTGTC	360
	lyGluValL	euGlnMatGl	uAspAspLeu	ValIleSerP	heGlnLeuMe	tLeuCysVal	(222)
	CTTGACTATT	TTATTAAACT	CTCACCTCCC	ATGTTGCTCA	AAGAACCATA	TAGTAAGTAT	420
	LeuAspTyrP	heIleLysLe	uSerProPro	MatLeuLeuL	ysGluProTy	rL	(240)
	TTAATTTTATG	CCCCTTTTAC	TTTCTCATTG	AGCAGTTGCT	TATTGAATGT	CTAGTGGGTA	480
	CCAAACATGG	TTCTAAGGCT	GACAGGATGA	TAAAAAATAA	ATCAgacatg	gactttggcc	540
	ataagtagtg	taagttatag	aaggaaagat	aagacatgga	aacaaatgat	tagagtata ^t	600
	ggtagaaagt	ggtttcgggt	caaaatacaa	caaatggagg	tttgggagac	aagaag	656
	--1.8 kb--						

09387483-083199

Fig. 6-3

EXON 8		gctattccat	gccttctctt	tgtatttggt	tatgagactg	tagtttacag	ttctttttgg	060
		gannagagta	gaagagggat	gCAAAACTA	ATATTAGTAC	ATAATTTGTA	GTAGATATGG	120
		ATGAAATTGT	TATCCTTCTA	ATGAAACCTA	ATAAGTAAAA	GTAGTAGAAT	GTTACCAAGA	180
		TTATTTTGA	CCTAAGTTAT	AGTTAGAATA	CTTCATTATT	TTATATGATG	GATGTACAAT	240
		TGTTCTTATC	TAATTTACCA	CTTTTACAGA	AACAGCTGTT	ATACCCATTA	ATGGTTCACC	300
				y	sThrAlaVal	IleProIleA	snGlySerPr	(250)
		TCGAACACCC	AGGCGAGGTC	AGAACAGGAG	TGCACGGATA	GCAAAACAAC	TAGAAAAATGA	360
		oArgThrPro	ArgArgGlyG	lnAsnArgSe	rAlaArgIle	AlaLysGlnL	euGluAsnAs	(270)
		TACAAGAATT	ATTGAAGTTC	TCTGTAAAGA	ACATGAATGT	AATATAGATG	AGGTAATTTA	420
		pThrArgIle	IleGluVail	euCysLysGl	uHisGluCys	AsnIleAspG	lu	(287)
		ACTTCATGAT	TTCTTTAAAA	CAGTTAAAGT	AGATTTAGAT	GTAAGTTCTC	CCTAACAAATA	480
		TTTACTTCTT	TTGTTATGAG	CATGTTTTTT	TTGTAATTAG	TGCTAACTCT	TTTGCAGTAG	540
		CAAAATATTT	AGAAAAAtta	attogttata	tttagttact	ttgatttaag	agagtagetc	600
		cctcactct						609
--1.8 kb--								
EXON 9		aagcattgaa	gctgtaatgc	atgtgattgc	acctgtgaat	agccactaca	cttcagccta	060
		ggcaatatag	agagaccct	tctcTAAGAA	AATAATAAAA	AATAAAAAAG	TTATACACAG	120
		ATTTTTTACT	GCATGGGGGA	TTGACACCTC	TAAGTTACCC	TGCATTGTTT	AAGAGTCAAG	180
		AGATTAGATT	TTGTTTAAA	TTTAAATGAT	CATGTTGTAA	CTTCATCTTT	TTCAGGTGAA	240
<u>EcoRI</u>							Vally	(289)
		AAATGTTTAT	TTCAAAAATT	TTATACCTTT	TATGAATTCT	CTTGGACTTG	TAACATCTAA	300
		sAsnValTyr	PhelYsAsnP	heIleProPh	eMetAsnSer	LeuGlyLeuV	alThrSerAs	(309)
		TGGACTTCCA	GAGGTAATCT	GAAAGGAAAT	TTAATAAAAT	ATTAATGTTT	TGAGACTGTG	360
		nGlyLeuPro	Glu					(313)
		GAGGGAGGAT	AATTGTCTAA	CTTTCTTAGA	TCAATTTACT	GTGTATCACA	TTTTTTTTTT	420
<u>HindIII</u>		GCCCAAGAAG	AATCTAGCCA	AGTAGAATTG	TGGTGAAACT	AACTTTGTGA	TAGTAacaaa	480
		aagctt						486
--1.9 kb--								
EXON 10		gtagcattgg	ctatctttgt	ctacataaaa	ttctaataaa	tattttctat	gcacgAAATA	060
		GACCTAAAT	CAAAGTTGAA	CAAATGTTGC	AATTTTCTGT	ACCTCACTTT	TAGATAGACC	120
		TTATTTATAT	TGCATGCGAA	CTCAGTGTAT	ATTACAAAAT	TAAATGTATA	TTATACAAAA	180
		ATTCTTTAAT	GAAATCTGTG	CCTCTGTGTG	CTGAGAGATG	TAATGACATG	TAAAGGATAA	240
		TTGTCAGTGA	CTTTTTTCTT	TCAAGGTTGA	AAATCTTTCT	AAACGATACG	AAGAAATTTA	300
<u>XbaI</u>				ValG1	uAsnLeuSer	LysArgTyrG	luGluIleTy	(325)
		TCTTAAAAAT	AAAGATCTAG	ATGCAAGATT	ATTTTGTGAT	CATGATAAAA	CTCTTCAGAC	360
		rLeuLysAsn	LysAspLeuA	spAlaArgLe	uPheLeuAsp	HisAspLysT	hrLeuGlnTh	(345)
		TGATTCTATA	GACAGGTATT	GCACATGGTA	TATTTGATTG	ATTTGCTTTA	GATATAGGTT	420
		rAspSerIle	AspSe					(350)
		GATACTGATA	TAGGTAGATT	ATATAGTCTT	TAGCTTAGTG	ACCTTTAGAT	ATCATTTATA	480
		ACAAATTACT	TTCAAATGTC	TTTATACAAA	GAAAAGTTTA	ACAGTATTTT	AAGcatataa	540
		cttatctaca	aatatagatt	taatgtgaat	tgtgtgtcct	ataacagtta	ccttttttnc	600
		gttaactgaa	tataattttt	aaaatgtgca	ccaaaagata	atggcta		647
--1.0 kb--								

0015128660

[illegible]

EcoRI

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-70.0 kb--

Fig. 6-6

EXON 18	atttttcta	ataagcg	tgttg	aagggtat	ac	atttttctac	ttttttgtgt	gtgggaagta	060
	caaaaattgt	CAATTGGGAA	TTTCGAAGTA	GAGAAAAATA	TTTCATTCTG	ACTTTTAAAT		120	
	TGCCACTGTC	AATTGTGCCT	AAAATTCATA	GTACTTACCA	TGTCAAACAA	TATGATTTTG		180	
	ATATGTACCT	GGGAAAATTA	TGCTTACTAA	TGTGGTTTTA	ATTTTCATCAT	GTTTTCATATA		240	
	GGATTACCT	TTATTTGATC	TTATTAAACA	ATCAAAGGAC	CGAGAAGGAC	CAACTGATCA		300	
	AspSerPro	LeuPheAspL	euIleLysG1	nSerLysAsp	ArgGluGlyP	roThrAspHi	(585)		
	CCTTGAATCT	GCTTGTCCCT	TTAATCTTCC	TCTCCAGAAT	AATCACACTG	CAGCAGATAT		360	
	sLeuGluSer	AlaCysProL	euAsnLeuPr	oLeuGlnAsn	AsnHisThrA	laAlaAspMe	(605)		
	GTAAGGAAAA	TATATGTTAT	GTTGACCATC	AAACTGCAAA	TAGATTTTAA	GCATAAGTGC		420	
	AATGTAACAT	TCTATAAAGA	CTGTAGGG	AATAGAATTT	TGAATAAGA	TAGTTTCTGT		480	
	TTTTAAGAAA	TTAGTAATAA	AAAGTACATg	acccaaataa	agtcataataa	aagagtacag		540	
	agtgtactactg	aatcacctag	gatttgcata	atgagagcag	ttttcatgg			589	
	--3.0 kb--								
EXON 19	tgtttttaag	ctggaatcac	cttatggctct	caataccact	ataattatta	aaattgTACA	060		
	TTATACATAT	ATAGCTATTT	TTTTCTAATA	AGGCAGTAAT	CCCCAGGAAA	AGCCATTAT	120		
	TAAAATAGAA	TTAGATATGA	TGATGACAAG	CAGTTTTCT	ATTAATATAT	CTTTCCAGC	180		
	TTGCATTTAA	ATAGTCTGCT	ATAATACCAA	TTAAATAGAC	AAGATGTATC	TGGGTGTACA	240		
	ACCTTGAAGT	GTATGTATAA	TCTGTGATT	TTAGCCAAC	TGAAATGAAG	ACTTTTCCTT	300		
	TAAATATATC	TAGGTATCTT	TCTCTGTAA	GATCTCCAAA	GAAAAAAGGT	TCAACTACGC	360		
		tTyrLeu	SerProValA	rgSerProLy	sLysLysGly	SerThrThrA	(621)		
	GTGTAAATTC	TACTGCAAT	GCAGAGACAC	AAGCAACCTC	AGCCTTCCAG	ACCCAGAAGC	420		
	rgValAsnSe	rThrAlaAsn	AlaGluThrG	lnAlaThrSe	rAlaPheGln	ThrGlnLysP	(641)		
	CATTGAAATC	TACCTCTCTT	TCACTGTTTT	ATAAAAAAGG	TTAGTAGATG	ATTATTTTCA	480		
	roLeuLysSe	rThrSerLeu	SerLeuPheT	yrLysLysV			(654)		
	AGAGCATGGA	CTCTGAAACT	AGGCTGACTG	GGTTCAAATC	ATGTTTCTTC	TACTTTCTAG	540		
	GTACATTACT	GGGCAAGTCA	CTTAATATCT	CTGTGTCTCA	GTTTCTCcat	ctataaaatg	600		
gaaatgataa	tgttgcgaga	tctttcttga	ctattcagag	tcgttttctg		650			
--2.8 kb--									
EXON 20	aaggaaaatc	catgeccent	cgggacatgc	ctgnccctctg	catttcttcca	tctgtatccc	060		
	ttgtaatatg	cctcataata	aaccagtaaa	catGTTTCTC	TGGGGGAAAG	AAAAGAGTGG	120		
	TAGAAAAGAG	GTTTCTGTTA	AAATGCTACT	TAACAGCATT	ATAATTAGTG	TAATTTTCATG	180		
	ATTTGAAAAA	AATCTACTTG	TAATTCAAAA	TGAACAGTAA	AAATGACTAA	TTTTTCTTAT	240		
	TCCCACAGTG	TATCGGCTAG	CCTATCTCCG	GCTAAATACA	CTTTGTGAAC	GCCTTCTGTC	300		
		al TyrArgLeuA	laTyrLeuAr	gLeuAsnThr	LeuCysGluA	rgLeuLeuSe	(671)		
	TGAGCACCCA	GAATTAGAAC	ATATCATCTG	GACCCTTTTC	CAGCACACCC	TGCAGAATGA	360		
	rGluHisPro	GluLeuGluH	isIleIleTr	pThrLeuPhe	GlnHisThrL	euGlnAsnG1	(691)		
	GTATGAACTC	ATGAGAGACA	GGCATTTGGA	CCAAGTAAAG	AAATCAAGCA	CTTCACCTTC	420		
	uTyrGluLeu	MetArgAspA	rgHisLeuAs	pGln			(702)		
	TCTCCTCCCT	ACTTACTTGT	TAAGTATTTT	CTTCTTTTCT	TTCTTTCTTT	CTTCTTTTCT	480		
	TTCTTTCTTT	CTTCTTTTCT	TTCTTTTCTT	TCTTTTCTTT	CTTCTTTTCT	TTCTTTTCTT	540		
	TTTTTTTGAG	ATAGAGTCTC	ACTCTGTTAC	CCAGGCTGGA	GTGCAGTGGC	GCAATCTCGG	600		
CTCACTGCAA	CCTCCGCCCTC	CCAGGTCAAG	TGATTCTCCT	GCCTCAGCCT	ccnaggagct	660			
aggatacagg	cgtgtaccac	cacacctgtg	taatttttgt	tatttagtag	agacagg	717			
--4.0 kb--									

Fig. 6-7

	caagagccaa	agttagggtg	atttacaac	caggtgatca	gtcctggata	attgagcctt	060
	ggtgatttgC	ATTTTGTTCT	TTAAACACAC	TTTGGGTAA	ACACTTCATG	TAGACTTTCA	120
<u>SacI</u>	AACTGAGCTC	AGTATGGA	GAAATAACTC	TGTAGATTAA	ACCTTTCTTT	TTTGAGGCTA	180
	AAAGAAAGAA	AATGGTATTT	TTTAAGAACA	AAACCATGTA	ATAAAATTCT	GACTACTTTT	240
EXON 21	ACATCAATTT	ATTTACTAGA	TTATGATGTG	TTCCATGTAT	GGCATATGCA	AAGTGAAGAA	300
		I	leMetMetCy	sSerMatTyr	GlyIleCysL	ysValLysAs	(716)
	TATAGACCTT	AAATTCAAAA	TCATTGTAAC	AGCATACAAG	GATCTTCCTC	ATGCTGTTCA	360
	nIleAspLeu	LysPheLysI	leIleValTh	rAlaTyrLys	AspLeuProH	isAlaValGl	(736)
	GGAGGTAGGT	AATTTTCCAT	AGTAAGTTTT	TTTGATAAAT	CCATATCCAT	AACATAACAT	420
	nGlu						(737)
	AGGTAATTCA	TTTGATCTCA	TTTATCATT	ATGAGATCAT	ATATTCTGTC	TGACCTTATT	480
	ATGTAAATTC	ACAAATAAAA	ACTTTTATAT	TATTTATTTG	TAACTTAAAT	AGAATTGGAA	540
	AGATAAGGGT	AATTATGAAA	TTACCCATAT	CATAGTTTTT	TATAAAGTTA	ATAAATAATA	600
	TTTTATCCCT	GTAATAAGCA	GGTATTTGTa	ataaacttga	catgagtcac	agaacattag	660
	atatcttgag						670
				--0.2 kb--			
<u>XbaI</u>	tccatctgct	gctgcctgge	tatttctctc	aatcgattct	gtgacatttc	acttctagaa	060
	gagcAGCTAT	AATCCAAGCC	TAAGAAGTAA	TTTTATTTAT	TTATTATTTT	TTCTTTTATA	120
	ATATGTGCTT	CTTACCAGTC	AAAAAGTATT	ATAAACTATT	AGAAAAGAAA	ATCTAAAGGT	180
EXON 22	AGAAATTTTA	AAATTCATTT	AACAAGTAAA	TTTTACTTTT	TTTTTTTTTT	TTTTTTTTTT	240
	ACTGTTCTTC	CTCAGACATT	CAAACGTGTT	TTGATCAAAAG	AAGAGGAGTA	TGATTCTATT	300
		ThrPh	eLysArgVal	leuIleLysG	luGluGluTy	rAspSerIle	(752)
	ATAGTATTCT	ATAACTCGGT	CTTCATGCAG	AGACTGAAAA	CAATATTTT	GCAGTATGCT	360
	IleValPheT	yrAsnSerVa	lPheMetGln	ArgLeuLysT	hrAsnIleLe	uGlnTyrAla	(772)
	TCCACCAGGG	TAGGTCAAAA	GTATCCTTTG	ATTGGAAAAA	TCTAATGTAA	TGGGTCCACC	420
	SerThrArg						(775)
EXON 23	AAAACATTAA	ATAAATAATC	TACTTTTTTTG	TTTTTGCTCT	AGCCCCCTAC	CTTGTCACCA	480
					ProProTh	rLeuSerPro	(781)
	ATACCTCACA	TTCTCGAAG	CCCTTACAAG	TTTCCTAGTT	CACCCTTACG	GATTCTTGGA	540
	IleProHisI	leProArgSe	rProTyrLys	PheProSerS	erProLeuAr	gIleProGly	(801)
	GGGAACATCT	ATATTTCAAC	CCTGAAGAGT	CCATATAAAA	TTTCAGAAGG	TCTGCCAACA	600
	GlyAsnIleT	yrIleSerPr	oLeuLysSer	ProTyrLysI	leSerGluGl	yLeuProThr	(821)
	CCAACAAAAA	TGACTCCAAG	ATCAAGGTGT	GTGTTTTCTC	TTTAGGGAAG	TAGTAAAGAA	660
	ProThrLysM	etThrProAr	gSerAr				(830)
	TGAGAGGGGG	ATTATTTTGA	TCCAAGAATA	AAAAATATAA	AGCATTCTTC	ATTTCAAATA	720
	AGCTAGACTC	TTGAAACTCT	ATTGCTTAT	TTAAGTAACA	TAATAAGAAT	ATGGGGGGGG	780
	GGTGAAAGAA	ATCTATTTAC	GACTTAAGCA	ACGCAAGATG	GCCGAATAGG	AACAGCTCCg	840
	gtctacagct	cccagcgtga	gcacgcagaa	gacgggtgat	ttctgcattt	ccatctgagg	900
	taccgggttc	atctcactag	ggagtgccag	acagt			935
				--7.4 kb--			
	ttgataactt	accattgat	ttatgaagaa	ctaagtaggg	gtaaccttga	aacttgccct	060
	tgccctccct	aaatatgggc	aatggcagna	tatgttcttg	cagacctata	acttttgctT	120
	TAAACTAAG	AGACTAGGTG	AGTATATGAT	TAGACGGGCA	CTGTTAGAAT	AATCCCAAAA	180
	TGAATATAGT	TTGTCAGTGG	TTCTAGGGTA	GAGGTAACTT	TTAATTTGGT	ATTCCTAATA	240
EXON 24	GTTCAGAATG	ATGTATTTAT	GCTCATCTCT	GCAAAATTGT	ATATGGTTTT	TTATTACTAA	300
	TTGGTATTTT	ATCTTAACTT	GACAGAATCT	TAGTATCAAT	TGGTGAATCA	TTCGGGGTGA	360
			gIleL	euValSerI	eGlyGluSer	PheGly	(840)
<u>HindIII</u>	GTATTTTCTT	TCTATGAAAT	ATAATAGTAT	GCATTGTAAG	TATAAAAGAA	ATTAAGCTT	420
	TCTATAATTT	GAATTTCCAA	ATGCAGTTAT	TCAAACACCT	CATCCAGGCA	TATTGCATAG	480
	AATTTTATGA	GATATATATA	TCTCAGATTT	ACTTTCAAAT	CAAGTTTAAT	CTCAAATCAT	540
	ACTCCTAATT	GGTGAACCTC	AAAACCTTTT	TAAATATCCA	CTTGAGATTA	TATAATACAT	600
	ATATACATTT	GTGTATATAG	ATACATATAT	ACGTGAGCTG	TTTTTGCTCA	CAACATTTCT	660
	ATCACCAAAT	GTGTGAGATT	TTTTTCTCAC	CCAAATGTAT	TCTTcaactc	tctgggtgctt	720
	ctacaattca	attcaattct	gacactaatt	accagag			758
				--2.8 kb--			

Fig. 6-8

EcoRI EXON 25	888gatggaa	ttaggtagtt	attctgattt	ttAGATTTTT	CATATCTTTT	ATTGTTGCCA	060	
	ATGAAGCAGA	AAATTTAAAT	GAAGTTATTA	CCTTTGCCCTG	ATTTTTGACA	CACCTCAAAC	120	
	TATAACTTGA	GGTTGCTAAC	TATGAAACAC	TGGCATTTAA	TGATTTAAAG	TAAAGAATTC	180	
	TGTAATTTGT	AGACTTCTGA	GAAGTTCCAG	AAAATAAAATC	AGATGGTATG	TAACAGCGAC	240	
		ThrSerG1	uLysPheGln	LysIleAsnG	lnMetValCy	sAsnSerAsp	(856)	
	CGTGTGCTCA	AAAGAAGTGC	TGAAGGAAGC	AACCCTCCTA	AACCACTGAA	AAAACCTACGC	300	
	ArgValLeuL	ysArgSerAl	aGluGlySer	AsnProProL	ysProLeuLy	sLysLeuArg	(876)	
	TTTGATATTG	AAGGATCAGA	TGAAGCAGAT	GGAAGGTAGG	AACCAGTTTT	GAATGTTTTTC	360	
	PheAspIleG	luGlySerAs	pGluAlaAsp	GlySe:			(888)	
	CAGTAGCTGA	GATGGTCATC	TGGGGAATCC	AGAGTCTCAG	CACTGCTCCT	GGCTTATACC	420	
EXON 26	AATTTCTTTC	ATGCCAAGTT	TATTTGGAAG	TTGTGAGAAT	GGCTCAAAAT	AATAGATATG	480	
	AGTGTAGTGC	AAAGTTAAAA	ACATCTTACA	AATTGCATAC	CAACATTCAG	TGAAGATATC	540	
	TAATAAACCC	TGATCTTTTT	TACAAAGCTA	TTGATAAAAT	TTTGTTTATTC	TTAACATTAA	600	
	ATTTAAAAAT	GTTTACTCTT	GAAAAATATT	AACCACTGTA	TTTTGTGAGA	ACCACTGAAA	660	
	AAATACATAG	CATCATAAAT	TTGTGACATT	TATGTTTTAG	ATGGTTAGTT	TTTAAATTTT	720	
	AAAATTAATA	GCTACTCACT	AAAATAATAG	CATAAAGTAA	GTCATCGAAA	GCATCATAGT	780	
	TACTGGAAAT	TTGAGTTTTC	CATTTATAAA	TACACATGAA	ATGTTTTGCA	TTTTTTTAAAT	840	
	CTGCAGTAAA	CATCTCCCAG	GAGAGTCCAA	ATTTACAGCAG	AAACTGGCAG	AAATGAGTAA	900	
		rLys	HisLeuProG	lyGluSerLy	sPheGlnGln	LysLeuAlaG	luMetT	(905)
	GTACTTTTTT	CACCTTGTGT	AAACGAAATA	AACAATTGTT	TACACTGCAA	GAAGTCTTTT	960	
EXON 27	CGTTATATAA	AAGAATGTAT	AATTTCTTCA	GTTGGCAGGT	TTGTTTATGC	ATTTAAAATA	1020	
	TAATTCATATC	AAGGTTATTT	ATCTACAAAC	ATTTGTGGAT	TAAATGTATG	ATGTAAAATG	1080	
	AAGGTCATTT	TTACCCTTTC	TATGATCTTT	CATGCAGGAA	GACTAAGAAG	TGAAACATTG	1140	
	CTTGACCACA	TTCAAcacaa	atggctacag	ttagaaaata	cttttagcaga	actacaaaga	1200	
	ggaaactat	gggagtgtta	gatataggga	aaagttttat	aaacctagca	tatgtaaaac	1260	
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	--1.7 kb--							
	tctagctatt	tgaatatgca	gtaaattaac	tgtaactcct	acgggtactgt	caaatactag	060	
	aatgaagacc	acctcttttt	gcAAGGTCCT	GAGCGCCATC	AGTTTGACAT	GAGCATAATA	120	
	TATATGGCAG	CCACTTGCCA	ACTTACCCAG	TACCATCAAT	GCTGTTAACA	GTTCTTCATC	180	
CTTTTTCCAG	CTTCTACTCG	AACACGAATG	CAAAAGCAGA	AAATGAATGA	TAGCATGGAT	240		
	hrSerThrAr	gThrArgMet	GlnLysGlnL	ysMetAsnAs	pSerMetAsp	(921)		
ACCTCAAACA	AGGAAGAGAA	ATGAGGATCT	CAGGACCTTG	GTGGACACTG	TGTACACCTC	300		
ThrSerAsnL	ysGluGluLy	sEND 3' untranslated	region begins here			(928)		
TGGATTCAAT	GTCTCTCACA	GATGTGACTG	TATAACTTTC	CCAGGTTCTG	TTTATGGCCA	360		
CATTTAATAT	CTTCAGCTCT	TTTTGTGGAT	ATAAAATGTG	CAGATGCAAT	TGTTTGGGTG	420		
ATTCCCTAAG	CACTTGAAAT	GTTAGTCATT	GTTATTTATA	CAAGATTGAA	AATCTTGTGT	480		
AAATCCTGCC	ATTTAAAAAG	TTGTAGCAGA	TTGTTTCCTC	TTCCAAAGTA	AAATTGCTGT	540		
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GAATCTGATA	TACTGTGTGC	TTGTTTTATA	AAATTTTGCT	TTTAATTTAA	TAAAAGCTGG	900		
AAGCAAAGTA	TAACCATATG	ATACTATCAT	ACTACTGAAA	CAGATTTTCAT	ACCTCAGAAAT	960		
GTAAAAGAAC	TTACTGATTA	TTTTCTTCAT	CCAACCTATG	TTTTTAAATG	AGGATTATTG	1020		
ATAGTACTCT	TGGTTTTTAT	ACCATTTCAG	TCACCTGAAT	TATAAAGTAC	CCATCTAGTA	1080		
CTTGAAAAAG	TAAAGTGTTT	TGCCAGATCT	TAGGTATAGA	GGACCCTAAC	ACAGTATATC	1140		
CCAAGTGCA	TTTCTAATGT	TTCTGGGTCC	TGAAGAATTA	AGATACAAAT	TAATTTTACT	1200		
CCATAAACAG	ACTGTTAAT	ATAGGAGCCT	TAATTTTTTT	TTCATAGAGA	TTTGTCTAAT	1260		
TGCATCTCAA	AATPATTTCTG	CCCTCCTTAA	TTTGGGAAGG	TTTGTGTTTT	CTCTGGAATG	1320		
GTACATGTCT	TCCATGTATC	TTTTGAACTG	GCAATTGTCT	ATTTATCTTT	TATTTTTTTA	1380		
AGTCAGTATG	GTCTAACACT	GGCATGTTCA	AAGCCACATT	ATTTCTAGTC	CAAAATTACA	1440		
HindIII	AGTAATCAAG	GGTCATTATG	GGTTAGGCAT	TAATGTTTCT	ATCTGATTTT	GTGCAAAAGC	1500	

0937430034860

Fig. 6-9

EXON 27
(CONTD)

TTCAAATTAA AACAGCTGCA TTAGAAAAAG AGGCGCTTCT CCCCTCCCCT ACACCTAAAG 1560
3' untranslated region (continued)

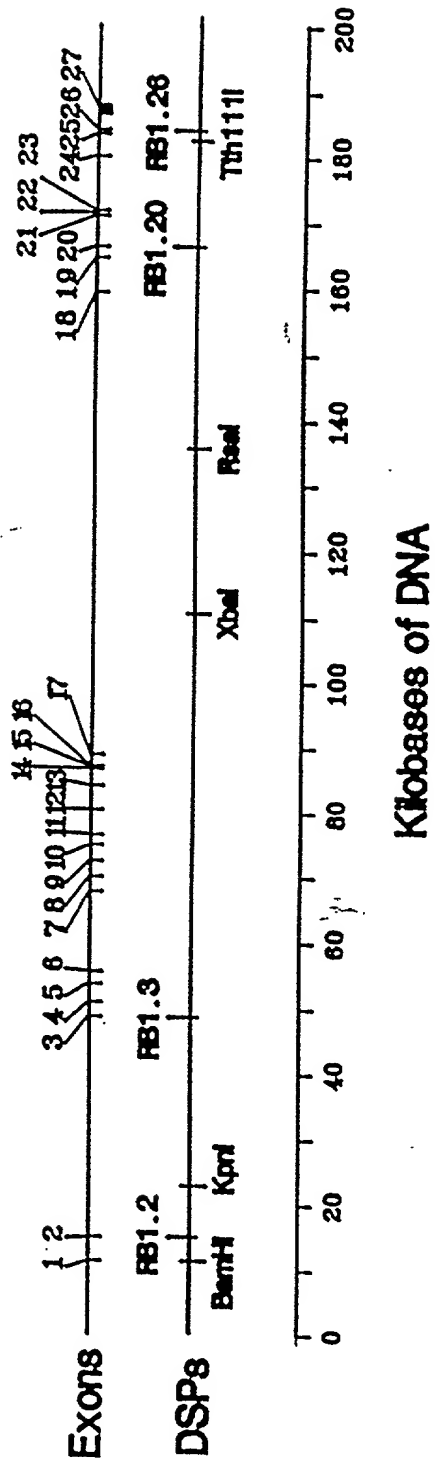
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TTTTAAAAAG AAATCTGGTC TTGTTAGAAA AAAAAATTTT ATTTTGTGCT CATTTAAGTT 1740
TCAAACCTAC TATTTTGACA GTTATTTTGA TAACAATGAC ACTAGAAAAC TTGACTCCAT 1800
TTCATCATTG TTTCTGCATG AATATCATAC AAATCAGTTA GTTTTTAGGT CAAGGGCTTA 1860
CTATTTCTGG GTCTTTTGCT ACTAAGTTCA CATTAGAATT AGTGCCAGAA TTTTAGGAAC 1920
TTCAGAGATC GTGTATTGAG ATTTCTTAAA TAATGCTTCA GATATTATTG CTTTATTGCT 1980
TTTTTGTATT GGTAAAACT GTACATTAA AATTGCTATG TTAATTTT CTACAATTAA 2040
TAGTTTGTCT ATTTTAAAT AAATTAGTTG TTAAGAGTC TTAATGGTCTG ATGTTGTGTT 2100
polyadenylation signal sequence polyadenylation site

CTTTGTATTA AGTACACTAA TGTTCTCTTT TCTGTCTAGG AGAAGATAGA TAGAAGATAA 2160
CTCTCCTAGT ATCTCATCCA TTCCTAGCCT TTAAGGGGCT CTATATGCTA GAGATTTCCA 2220
AATTTATTTT TTCAGCCCTG ATCTTTTCAC AGAGGTCAAG Gcttttatag ccaacagaac 2280
tcttgattcc tactccentc tacccaatgt ctccaaatat aaactaaaat caaataaata 2340
aaaatccttt tt 2352

00307450.003499

Fig. 7

HUMAN RETINOBLASTOMA GENE



17 June.

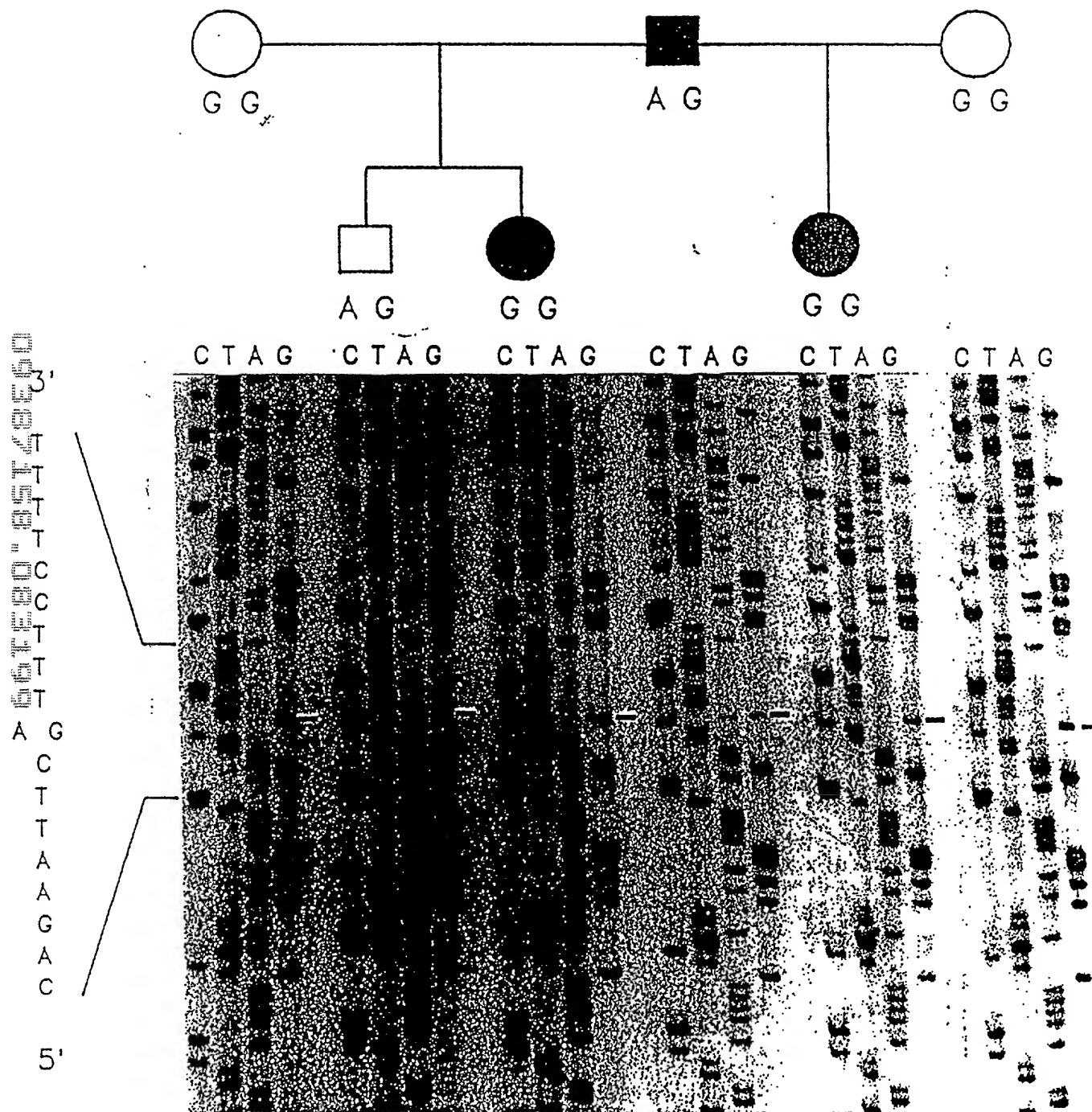
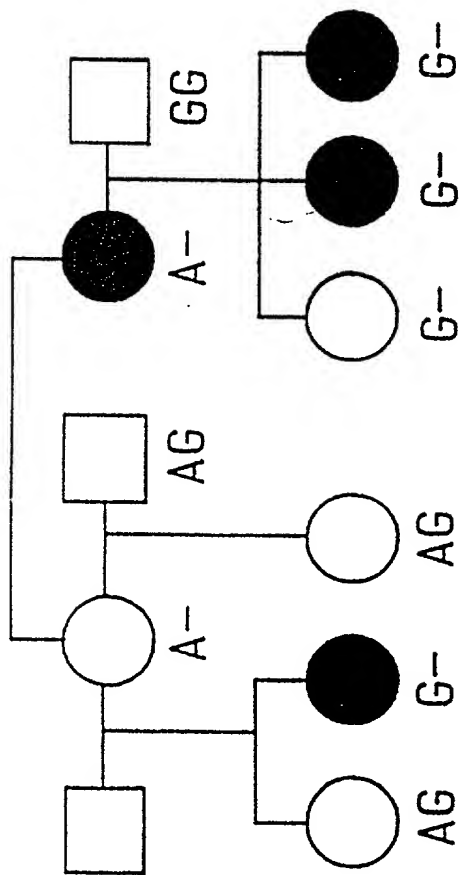
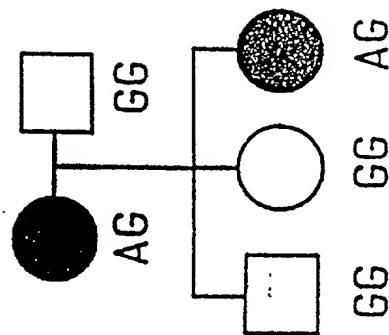


Fig. 9

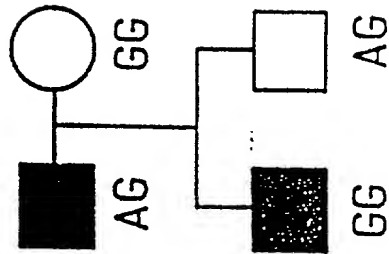
RB-32



RB-36



RB-50



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Dryja, et al. Examiner: Y. Eyler
Serial No. : 08/255,572 Art Unit: 1806
Filed: June 8, 1994
For: DIAGNOSIS OF RETINOBLASTOMA

Assistant Commissioner for Patents
Washington, D.C. 20231

**TRANSMITTAL OF REVOCATION AND SUBSTITUTION
OF POWER OF ATTORNEY BY ASSIGNEES**

Enclosed for filing in the above-captioned application is a REVOCATION AND
SUBSTITUTION OF POWER OF ATTORNEY BY ASSIGNEES UNDER 37 CFR §§ 3.71 AND 3.73(B)
from the Massachusetts Eye and Ear Infirmary and the Whitehead Institute ("assignees")
revoking all previous powers of attorney pursuant to 37 C.F.R. § 1.36, and certifying pursuant to
37 CFR §§ 3.71 and 3.73(b) that to the best of their knowledge and belief they are together the
owners of the entire right, title and interest in this application. Please charge any fees or apply
any credits to our Deposit Account No. 50-0311, Ref. No. 19100-020.

Respectfully submitted,

Date: August 7, 1999

Leslie Meyer-Leon
Leslie Meyer-Leon
Reg. No. 37,381

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TRADOCS: 1230601.1 (qjd011.doc)

I hereby certify that this document is being deposited with
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attached, addressed to Assistant Commissioner for Patents,
Washington, D.C. 20231, on 08/06/99.

By: Billy D. Edwards

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Dryja, et al.	Examiner:	Y. Eyler
Serial No. :	08/255,572	Art Unit:	1806
Filed:	June 8, 1994		
For:	DIAGNOSIS OF RETINOBLASTOMA		

Assistant Commissioner for Patents
Washington, D.C. 20231

**REVOCATION AND SUBSTITUTION OF POWER OF
ATTORNEY BY ASSIGNEES UNDER 37 CFR §§ 3.71 and 3.73(b)**

As assignees of the entire interest in the above-identified application, the Massachusetts Eye and Ear Infirmary and the Whitehead Institute ("assignees") hereby revoke all previous powers of attorney pursuant to 37 C.F.R. § 1.36.

Pursuant to 37 CFR §§ 3.71 and 3.73(b), assignees certify that to the best of their knowledge and belief they are together the owners of the entire right, title and interest in and to the above-identified application. This application is assigned of record to Massachusetts Eye and Ear Infirmary, a Massachusetts corporation having a place of business at Boston, Massachusetts, by virtue of an assignment submitted to the Patent and Trademark Office for recording on April 24, 1989 at Reel 5081, Frame 0968 and 0969; and is assigned of record to Whitehead Institute, a Delaware corporation having a place of business at Cambridge, Massachusetts, by virtue of an assignment submitted to the Patent and Trademark Office for recording on April 24, 1989 at Reel 5081, Frame 0970.

I hereby certify that this document is being deposited with the United States Postal Service with first-class postage attached, addressed to Assistant Commissioner for Patents, Washington, D.C. 20231, on 08/06/99.

By: *Belinda Edwards*

Further pursuant to 37 CFR §§ 3.71 and 3.73(b), assignees hereby appoint the following to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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Signed on behalf of:
THE MASSACHUSETTS
EYE AND EAR INFIRMARY



F. Curtis Smith
President

Date: 7/22/99

Signed on behalf of:
THE WHITEHEAD INSTITUTE



John Pratt
Vice President

Date: 7/26/99

TRADOCS: 1224943.1 (q967011.doc)